Synthesis and Structure Activity Relationship Studies of a Natural Product-Derived Compound Library

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SYNTHESIS AND STRUCTURE ACTIVITY RELATIONSHIP STUDIES OF A
NATURAL PRODUCT-DERIVED COMPOUND LIBRARY

By

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Dissertation

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Infectious diseases will always be a public health concern. The incidence of bacterial strains found in clinical settings that exhibit resistance to our current arsenal of antimicrobial drugs has risen sharply in the past twenty years and is projected to keep rising. The Infectious Diseases Society of America (IDSA) stated that more people die of methicillin-resistant *Staphylococcus aureus* (MRSA) infections per year in US hospitals than HIV/AIDS and tuberculosis combined. However, stagnation of research in the area of infectious diseases over the past forty years has left us dreadfully behind in the battle against these deadly pathogens.

Recently, researchers have started getting back to the roots of drug discovery by screening possible antibiotic-producing organisms isolated from the environment. Unfortunately this will take time as many of the more common antibiotics have already been found in this manner, leaving the rarest compounds to still be found. Other drug discovery programs focus on the production of large chemical libraries by combinatorial synthesis. While hundreds of thousands of compounds have been made in this way, only one FDA approved drug has been made by de novo synthesis. Compounds made by combinatorial methods lack much of the complexity afforded by natural products. Yet, it is the complexity of natural products that makes them good antibiotics.

The strategy we employ is to use natural products in the synthesis of combinatorial libraries. In this manner, we are able to incorporate complexity into a large number of compounds, increasing our chances of finding a new compound with antimicrobial activity.

Herein is described a compound library derived from the natural product, nonactin. A preliminary library was previously synthesized by Phillips that yielded two lead compounds that were the starting points for this work. The lead compounds were systematically modified over four iterations of a Structure Activity Relationship study in an effort to maximize antimicrobial activity. Antimicrobial activity of the compounds from the SAR study against *Bacillus subtilis* was increased nearly 200-fold from the initial leads and spectrum of activity was expanded to include *Staphylococcus aureus* and *Enterococcus faecalis*. 
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I would like to thank first and foremost my advisor, Dr. Nigel Priestley, for his support, help and guidance through the years and for giving me the opportunity to work in his lab. I would also not be here without the personal touch of Dr. Chris Palmer’s recruitment phone call all those years ago when I was searching for the school I would call home for the next six years. My studies also would have been much more difficult without the support, understanding and help of co-workers and friends over the past few years. Each member of the Priestley Group that I have had the pleasure of working with has imparted some form of knowledge or life lesson to me that has helped me along the way. Most of my lab time here, however, I have shared with my co-workers Heather Ewing and Joshua Phillips. Our little lab family has shared many experiences and indeed, without their hard work and their help, I would not be here now. The faculty members of the Chemistry Department have always been helpful to and supportive of me and I would like to thank them for being very kind over the years. I would also like to give thanks to Roslyn Pinson and Janna Taylor for their parts in giving advice and support during the stressful times, of which there have been many. I give a very special thanks to Dave and Erin Bolstad for being my family away from home, for all of their help, care, encouragement, generosity and friendship. And finally, without the love and support of Mom, Dad and my family, I surely would not have been able to accomplish what I have over the past few years and would not be here now. Thank you for always being there and for always believing in me.
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List of Abbreviations

Ambient temperature = 23°C

ATR = Attenuated Total Reflectance

Calcd. = Calculated

CHCl₃ = Chloroform
\[ \text{CH}_2\text{Cl}_2 = \text{Dichloromethane} \]
\[ \text{cm} = \text{Centimeter} \]
\[ \text{cm}^{-1} = \text{Inverse Centimeters} \]
\[ d = \text{Day} \]
\[ d \text{ (in spectra descriptions)} = \text{Doublet} \]
\[ \text{dd} = \text{A Doublet of Doublets} \]
\[ \text{ddd} = \text{A Doublet of Doublets of Doublets} \]
\[ \text{DMF} = \text{Dimethyl Formamide} \]
\[ \text{dq} = \text{A Doublet of Quartets} \]
\[ \text{ESI} = \text{Electrospray Ionization} \]
\[ \text{EtOAc} = \text{Ethyl Acetate} \]
\[ \text{FAS} = \text{Fatty Acid Synthase} \]
\[ g = \text{Gram} \]
\[ H = \text{Hydrogen} \]
\[ h = \text{Hours} \]
\[ \text{hex} = \text{Hextet} \]
\[ \text{HRMS} = \text{High Resolution Mass Spectrometry} \]
\[ \text{Hz} = \text{Hertz} \]
\[ \text{IR} = \text{Infrared} \]
\[ M = \text{Molar} \]
\[ \text{MHz} = \text{Megahertz} \]
\[ \text{MIC} = \text{Minimum Inhibitory Concentration} \]
\[ \text{mL} = \text{Milliliter} \]
mmol = Millimole  
MS = Mass Spectrometry  
$m/z$ = Mass to Charge Ratio  
NaCl = Sodium Chloride  
NaHCO₃ = Sodium Bicarbonate  
NMR = Nuclear Magnetic Resonance  
NaOH = Sodium Hydroxide  
NH₄Cl = Ammonium Chloride  
PKS = Polyketide Synthase  
ppm = Parts per Million  
q = A Quartet  
quat = Quaternary Carbon  
quint = Quintet  
s = Singlet  
SAR = Structure Activity Relationship  
sec = Seconds  
t = A Triplet  
t-BuOH = tert-Butyl Alcohol  
THF = Tetrahydrofuran  
THP = Tetrahydropyran  
TLC = Thin Layer Chromatography  
All references to Na₂SO₄ and MgSO₄ refer exclusively to anhydrous Na₂SO₄ and MgSO₄
CHAPTER 1

THE HISTORY OF ANTIBIOTICS
An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms. The action of an antibiotic against micro-organisms is selective in nature, some organisms being affected and others not at all or only to a limited degree; each antibiotic is thus characterized by a specific antimicrobial spectrum. The selective action of an antibiotic is also manifested against microbial vs. host cells. Antibiotics vary greatly in their physical and chemical properties and in their toxicity to animals. Because of these characteristics, some antibiotics have remarkable chemotherapeutic potentialities and can be used for the control of various microbial infections in man and in animals.²

-Waksman, 1947

1.1 Antibiotic Beginnings

Antibiotics are chemical compounds that kill or hinder the growth of bacteria. They can be natural products made by plants and microorganisms in nature, chemically synthesized or made in a semi-synthetic manner which involves the chemical modification of natural products. The treatment of wounds and infections with natural products is not a recent idea. Many ancient cultures used plant materials to treat open wounds.³⁻⁵ Though there is a long and interesting history behind the eventual connection of disease with infectious agents, a more modern view of infectious disease and antibiotic treatment will be discussed within this work beginning in the 19th century with the advent of germ theory, which culminated in the famous broth experiment of Louis Pasteur in 1859.⁶

Pasteur observed and followed up on his observations that if a sterile nutrient broth was placed in a swan-necked flask, no growth occurred. He concluded that the growth was caused by organisms that were brought in on dust particles in the air and
that the torturous route that dust particles had to take through the swan-necked opening of the flask kept them from depositing the microorganisms in the broth. Germ theory was the antithesis of the popular theory of spontaneous generation and was under considerable debate at that time.\[^7\] \[^8\] Several subsequent experiments by Pasteur’s contemporaries---notably Roberts in 1874, Huxley and Tyndall, both in 1875---supported his work and furthered the field of microbiology.\[^8\] The collective observations of Pasteur’s three contemporaries constituted the earliest discoveries (at the time their “discoveries” were more observations in passing) of antibiosis. They were each working with different nutrient broths that were made by infusing water with the bodies of various organic (plant and animal) materials. Since they were each looking for evidence against spontaneous generation, their hypotheses were that sterilized broths would not be able to spontaneously grow microorganisms and become spoiled. As they still had not mastered (or invented) sterilization techniques, some broths became contaminated or had already contained microorganisms that were able to grow. It was in these samples that the first biological antagonistic effects were seen. The first observation was that the growth of *Penicillum glaucum* inhibited the growth of bacteria and that the presence of many bacteria in an infusion contrariwise would prevent the growth of the fungus. In 1874, Roberts described an observation of his experiments:

\[...it\ \textit{seemed, in fact, as if this fungus played the part of the plants in an aquarium, and held in check the growth of Bacteria, with their attendant putrefactive changes. On the other hand, the Penicillum glaucum seldom grows vigorously, if it grow[s] at all, in liquids which are full of Bacteria.}\[^9\]\]
This observation is the first known published result describing bioantagonism. The following year, Huxley and Tyndall exchanged correspondence in which Huxley ultimately describes a curious lack of growth of bacteria in a flask open to the air that became inoculated with Penicillium spores from the air.\textsuperscript{8} Tyndall performed similar tests and reported that:

\textit{[I]n every case where the [Penicillin] mould was thick and coherent the bacteria died, or became dormant, and fell to the bottom as a sediment. The growth of mould and its effect on the bacteria are very capricious.}\textsuperscript{8}

And that,

\textit{[An] absence of uniformity was manifested in the struggle for existence between the bacteria and the Penicillium. In some tubes the former were triumphant; in other tubes of the same infusion the latter was triumphant.}\textsuperscript{8}

In 1877, two years later, Pasteur commented that if the antagonism between microorganisms could be harnessed and humans could intervene in this struggle, it would offer “perhaps the greatest hopes for therapeutics.”\textsuperscript{10} It would be another fifty years before a similarly serendipitous observation by Alexander Fleming resulted in his discovery of penicillin and another ten before the proper research for its full development was done by Florey and Chain in 1940, ushering in the “golden age” of antibiotics that lasted for twenty years.\textsuperscript{11,12}
1.2 The Historical Development of Antibiotics

The golden age of antibiotics was the period from 1940 to 1960 (Figure 1.1), when microbiologists screened hundreds to thousands of natural compounds isolated from bacteria. This age saw the discovery and development of β-lactams, polyketides, aminoglycosides, phenylpropanoids, macrolides, glycopeptides and the synthetic (fluoro)quinolones. These structural classes represent the majority of the most successful antimicrobials, many of which are still in use today. The synthetic antibiotic, prontosil, was developed in 1932, after the initial discovery but before the full development of penicillin. Prontosil was the first of the sulfonamide drugs. These were used primarily for the treatment of puerperal fever caused by infection from the opportunistic Gram-positive bacteria, *Streptococcus pyogenes*. The glycopeptide, vancomycin was discovered in 1953 and is still used as a “drug of last resort” for Gram-positive infections.¹,¹⁴,¹⁵

The last class developed in the golden age was the quinolones, with the discovery of nalidixic acid in 1962.¹⁶ Forty years passed before a new structural class of antibiotic was discovered.¹⁷ The last completely new structural class discovered were oxazolidinone-containing compounds, which, like the sulfa drugs and quinolones, are fully synthetic. The first oxazolidinone antibiotic, linezolid, was FDA approved in 2000.

---

¹ Gram-positive and Gram-negative refer to the ability of the peptidoglycan layer of the cell wall to take up and retain the dye, crystal violet. Gram-positive bacteria have a thick peptidoglycan outer layer and retain the dye, whereas Gram-negative bacteria have a thin peptidoglycan layer surrounded by a second outer membrane and are unable to retain the dye. Peptidoglycan will be discussed further in Section 1.5.

¹⁷ In 1969, Surgeon General William H. Stewart made the painfully erroneous statement that “The time has come to close the book on infectious disease.”
Figure 1.1: Timeline of the development of the different classes of antibiotics. This timeline represents the year each structural type of antibiotic was approved or discovered. The first representative compound from each structural class is shown.
1.3 Diversity Within Antibiotic Structural Classes

There are several drugs found in each structural class of antibiotic. Some of them are naturally occurring relatives of the first compounds discovered in the class, such as benzylpenicillin, phenoxyethylpenicillin and cephalosporin C, all of which are “parent” β-lactams, which are the natural products isolated directly from the microorganisms that produce them, in this case fungi of the genera *Penicillium* and *Acremonium*. Others are semi-synthetic analogs of the naturally occurring parent compounds, such as methicillin, amoxicillin and cefotaxime all of which are made from the synthetic modification of a common precursor structure known as a scaffold (Figure 1.2)
Figure 1.2: Penicillin and cephalosporin derivatives. These compounds represent both naturally occurring (left) and semisynthetic members (right) of the $\beta$-lactam class of antibiotics.

Semi-synthetic analogs of the natural product parent compounds are made for several reasons: Of chief importance is to combat resistance that is inherent to the nature of antibiotics and the “survival of the fittest.” Each antibiotic class has its own mechanism of action and in some cases even drugs within one class have different mechanisms of action. The aminoglycosides, for example, block the translocation steps of protein biosynthesis but bind to different sites on the ribosome. If an antibiotic kills 99% of a population of bacteria but is unable to kill the remaining 1% because they are resistant to the antibiotic,$^*$ the susceptible bacteria are destroyed and the strong or unsusceptible bacteria are able to grow back in much higher numbers. Thus, the most

$^*$ Antibiotic resistance will be discussed more in depth in Section 1.6.
fit organisms are able to survive and an ever increasing number of antibiotic resistant strains are seen (Figure 1.3)

![The Rise of Antibiotic Resistance](image)

**Figure 1.3:** The rise of antibiotic resistance. This figure shows the steady increase of resistance seen in three major human pathogens: MRSA = methicillin resistant *Staphylococcus aureus*; VRE = Vancomycin resistant *Enterococci*; FQR = Fluoroquinolone resistant *Pseudomonas aeruginosa*. Percent incidence is the percentage of clinical isolates of the bacteria that exhibit resistance. Image reproduced from The Infectious Disease Society of America’s website with implied permission.

In stark contrast to this increasing trend in antibiotic resistance is the steady decline of new antibiotics that are being developed (Figure 1.4). New antibiotics are needed to combat the rise of resistance in common infectious pathogens. Resistance is intrinsic to the nature of antibiotics and harmful pathogens begin to develop resistance against new antibiotics the moment the drugs reach the clinic and in some cases, even before. For that reason alone, new chemical entities with antimicrobial activity will continue to be necessary until perhaps a newer therapeutic technology is developed.
Semi-synthetic derivatives are made in an effort to battle the increasing incidence of resistance, but there are limitations to these modifications.*

Figure 1.4: The number of new antibacterial agents from 1983-2007. A steady decline in the development of new antibacterial compounds can be seen over the past 25 years. Image reproduced from The Infectious Disease Society of America’s website with implied permission.

1.4 Antibiotic Targets

Antibiotic resistance has most likely co-evolved with the production of antibiotics from producing organisms.1 Antibiotic compounds are produced by microorganisms in nature. These compounds afford their producing organisms the ability to compete against other microorganisms for nutrients and territory in their habitats. The Actinobacteria phylum of bacteria is one of the largest taxonomic units.

* The limitations of semi-synthetic derivatives will be discussed in Section 1.7.
Streptomyces, the largest genus in Actinobacteria, are the most abundant life forms found in soil. Streptomyces are also responsible for the production of over two-thirds of all naturally occurring antibiotics. Since these bacteria live in a veritable soup of other microorganisms, it would make sense that they have evolved ways to eliminate competitors. In order to eliminate other organisms that are competing for resources, a vital system or pathway of the rival must be targeted and inhibited. To date, there are four major targets of antibiotics: 1) Cell wall biosynthesis, 2) Protein synthesis, 3) DNA repair and replication and 4) Folic acid metabolism. Each major class of antibiotics targets at least one aspect of one of these pathways (Table 1.1).

Table 1.1: Major Antibiotic Classes and Targets

<table>
<thead>
<tr>
<th>Antibiotic Class</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfonamides</td>
<td>Folic Acid Synthesis</td>
</tr>
<tr>
<td>β-lactams</td>
<td>Cell Wall Biosynthesis</td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
</tr>
<tr>
<td>Tetracyclins</td>
<td></td>
</tr>
<tr>
<td>Macrolides</td>
<td>Protein Biosynthesis</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td></td>
</tr>
<tr>
<td>Oxazolidinones</td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>DNA Replication and Repair</td>
</tr>
</tbody>
</table>

1.5 The Mode of Action of β-lactam Antibiotics

The mode of action of the β-lactams is probably the best understood and will be used here as a model system with which to explain the links between antibiotic modes of action, resistance, and the reasoning behind semi-synthetic modifications.
All β-lactam antibiotics, by virtue of their core structure, inhibit cell wall biosynthesis. This is accomplished through the inactivation of a transpeptidase enzyme that catalyzes the cross-linking of two strands of peptidoglycan by the covalent addition of the drug. Peptidoglycan is the main component of bacterial cell walls and is made up of a highly ordered, cross-linked system of sugars and peptides (Figure 1.5). The cross-linking of the peptide chains is what gives the cell wall its strength and protects the bacterium against osmotic pressure that could cause the cell to lyse.

Figure 1.5: A cartoon representation of four layers of peptidoglycan. Green and blue spheres represent N-acetylmuramic acid and N-acetylglucosamine, respectively. Yellow bars represent cross-linked peptide chains. Cartoon was taken from the Bug research group webpage, University of Warwick.²⁰

The transpeptidase enzymes that are responsible for catalyzing the cross-linking reaction belong to the family of serine hydrolases, which use the hydroxyl group of serine as a nucleophile for the breaking of the penultimate amide bond of the peptide...
chain (as opposed to a water molecule in a normal hydrolysis). The enzyme is then displaced from the peptide by nucleophilic attack from an amine on the neighboring peptide chain (Figure 1.6). A nearby, basic side chain of the transpeptidase is used as the conjugate base for the reaction.\textsuperscript{1,21}

The β-lactams mimic the structure of the D-alanyl-D-alanine dipeptide at the end of the section of peptidoglycan about to be cross-linked and bind to the active site serine of the transpeptidase before it is able to catalyze the cross-link reaction (Figure 1.7). Thus, the transpeptidase is covalently inactivated and must be hydrolyzed itself before it can resume its function. This regeneration of the enzyme takes several hours to days to occur and by the time the covalent inhibition is reversed, the cell wall has been weakened and the cell will not be able to divide properly. This combination of factors causes the cell to lyse.
Figure 1.6: Peptidoglycan crosslinking mechanism. A representation of one layer of a single unit of peptidoglycan and the basic mechanism of a cross-linking reaction. Reproduced from “Antibiotics.”\textsuperscript{1}
Figure 1.7: β-lactam inhibition. The covalent inhibition of the transpeptidase enzyme by a β-lactam antibiotic is shown. The enzyme-antibiotic complex is unable to catalyze the cross-linking reaction and is slow to hydrolyze due to the lack of available water molecules in the active site of the enzyme.

The mechanism shown (Figure 1.7) only represents a general mechanism of the β-lactam antibiotics. There are many different types of transpeptidases* that have widely varying roles in the metabolism and upkeep of the cell wall. This wide variety of roles gives an interesting insight into the development and evolution of bacterial resistance to the β-lactams.21, 22

* Transpeptidases are also called Penicillin-Binding Proteins or PBP.
1.6 Types of Antibiotic Resistance

There are two main types of resistance that bacteria can have against a drug. The first is to have a natural immunity. Some microorganisms simply don’t have a target for an antibiotic to affect. For example, fungi have a cell wall composed of chitin, which is a polysaccharide that is unlike peptidoglycan. Therefore, fungi do not have transpeptidase enzymes that can be targeted by the β-lactam antibiotics. Other microorganisms may be antibiotic producers that have co-evolved protection mechanisms in order to defend themselves from their own destructive chemicals. For instance, Gram-negative bacteria have a second outer membrane surrounding their less robust cell wall. This second, outer membrane contains efflux pumps that regulate a majority of the compounds that are allowed into the cell, including many antibiotic compounds. The second type of antibiotic resistance manifested in bacteria is acquired resistance, which is obtained from the surrounding environment or genetic mutation.¹

1.7 Natural Resistance to β-lactam Antibiotics

In the case of the β-lactams, enzymes known as β-lactamases co-evolved with the transpeptidases (PBPs) produced by the bacteria. The β-lactamases act as suicide enzymes to mimic PBPs and bind to the β-lactam antibiotics before they can inactivate the crucial PBPs involved in cell wall biosynthesis. The first β-lactamases were discovered shortly after the development of penicillin and are believed to have been
around even before its widespread clinical use, though their original function (if other than β-lactam antibiotic resistance) is yet unknown.\textsuperscript{22, 23}

Selective pressure from the widespread use of penicillin caused an increase of the production of several different types of β-lactamases which started to confer more and more resistance to the β-lactam antibiotics. This increase in resistance sparked the development of semi-synthetic analogs of penicillin.\textsuperscript{1} The synthetically modified antibiotics then instigated the production of more β-lactamases by the bacteria and thus began the continuing struggle between antibiotic development and resistance. The increased immunity from the selective pressure of the antibiotics starts to drift into the realm of acquired resistance, but the original resistance mechanism (i.e., the presence of β-lactamases) in this case is inherent to the bacteria.

Today, there are several generations and hundreds of semisynthetic β-lactam antibiotics which have been used for over 50 years.\textsuperscript{13} Each generation either combats resistance or increases the spectrum of activity to include other species of bacteria. This struggle is clearly seen in every known antibiotic class and is assumed, due to the nature of living organisms, to continue with new structural classes yet to be found.

Another type of natural immunity is the co-evolution of protective mechanisms within the producing organism. This is in contrast to the previous type because the antibiotic producer in the case of β-lactam antibiotics, originally \textit{Penicillium notatum}, a fungus, is a completely different species than the target organisms which are Gram-positive bacteria. The resistance mechanism against erythromycin, on the other hand,
arises from the producing organism itself. Erythromycin is a macrolide antibiotic that inhibits protein biosynthesis (Section 1.4) through non-covalent binding to the 23S subunit of the bacterial ribosome.\textsuperscript{1} The producing bacteria, \textit{Saccharopolyspora erythraea}, has a modification of the 23S ribosomal subunit which prevents the antibiotic from binding before it is exported from the producer.\textsuperscript{24, 25}

1.8 Acquired Resistance

Each type of natural immunity is based in the genomic makeup of the resistant species. However, microorganisms in close proximity to one another may perform horizontal gene transfers in order to maintain and propagate genetic diversity.\textsuperscript{26-28} Because of horizontal gene transfer, resistance mechanisms are able to be shared among bacteria that come in contact with one another. An increasingly common occurrence is that resistance mechanisms are seen in large islands in the genome or plasmids in resistant bacteria.\textsuperscript{14, 29}

Before the widespread use of antibiotics in the 1940s, the selective pressure for the buildup of resistance was low. In nature, there are not large reservoirs of bacteria in an environment where a wide variety of antibiotics are always present. However, in hospital settings where a high incidence of infections occur, many antibiotics are used and selectivity is very high for resistant strains of pathogens to propagate.\textsuperscript{30} The highly selective environment, high rates of bacterial replication (the doubling time of \textit{Staphylococcus aureus} is 60 minutes in vivo)\textsuperscript{31} and ease of horizontal gene transfer combine to create an ideal situation from which resistant bacteria can flourish. Indeed,
most of the highly resistant and virulent strains of MRSA and VRE originate in hospitals (Figure 1.3).

1.9 The Necessity for New Antibiotic Compounds

In order to combat this inevitable rise in resistance, new antibiotics are needed.\textsuperscript{30,32,33} Semi-synthetic analogs of existing antibiotic will always be a source of new compounds that may be used against resistant strains. But inevitably, the bacteria will evolve and develop resistance to them as well. What is really needed is a new structural class of antibiotic that has an entirely new mode of action which will be effective against strains of bacteria that already have resistance to current drugs. To find a new structural class of antibiotics, the search for new chemical entities must continue. An ideal way to search for a new structural class of antibiotic compounds would be to synthesize or isolate large numbers of structurally diverse chemical compounds and screen them for antimicrobial activity. Isolation of natural products has historically worked for discovering most of our current antibiotic compounds. But, it is thought that it has recently become much more difficult to find new antibiotic-producing organisms.\textsuperscript{34} Natural product screening programs are currently technologically limited to low throughput methods which tend to lead to the “re-discovery” of known antibiotics.\textsuperscript{34} Another problem with this method is that the antibiotics from the most commonly isolated microorganisms have already been discovered and new antibiotic compounds will likely be found in more uncommon microorganisms. Baltz noted that streptomycin can be found after screening one
hundred random actinomycetes found in the soil.\textsuperscript{34} Eli Lilly screened approximately five
million organisms before they discovered the lipopeptide, daptomycin, and likely, over
ten million microorganisms will have to be screened before the next clinically useful
drug will be found.\textsuperscript{34} So new, high throughput screening methodology will have to be
developed for that avenue of drug discovery to yield useful results. The difficulties of
natural product screening programs leave us to pursue the chemical synthesis of new
possible drugs. However, if chemical compounds are to be synthesized, there ought to
be some sort of rationale behind where to start.
CHAPTER 2

AN APPROACH TO THE GENERATION OF NEW ANTIBIOTICS
2.1 Chemoinformatic Analysis of Drug Sources

In 2003, Feher and Schmidt compared compounds found in combinatorial synthetic libraries, natural product libraries and approved drugs using a principal component analysis. They compared the three sets of chemical compounds based on over forty topological and structural parameters such as molecular weight, number of chiral centers, ratio of aromatic rings to ring atoms, number of hydrogen bond donors and acceptors and number of C-C, C-N, C-O and C-X bonds. The goal was to mathematically quantify and compare the structural and chemical diversity exhibited in each group of compounds.

Their analysis showed that the structural space occupied by the large set of combinatorial compounds (n = 670,000) was well defined and rather limited. In contrast, the structural space occupied by both natural products (n = 3,000) and drugs (n = 11,000) not only covers the space occupied by combinatorial compounds but also an additional area, indicating more structural diversity (Figure 2.1).

![Figure 2.1: A 2-D Plot of the first two principal components of Feher and Schmidt's chemoinformatics study. Each data point represents one individual compound. a) Synthetic Compounds (n = 670,000), b) Natural Products (n = 3,000), and c) Drugs (n = 11,000).](image-url)
The parameters concerning chirality and rigidity were among the most disparate when comparing natural products and drugs to synthetic libraries. Interestingly, the authors emphasize that while drug development programs are focused on making thousands of compounds quickly, the methods and reactions that larger pharmaceutical companies employ remove the very physiochemical characteristics that effective drugs possess, namely, number of chiral centers and overall structural rigidity of the compounds. A successful drug development program would ideally focus on producing large numbers of compounds that are complex enough to sample more chemical space than a simple combinatorial library.

2.2 Syntheses and Drawbacks of Synthetic Libraries

The fundamental difference reflected in the limited chemical space that is sampled by synthetic compounds is mainly due to the manner in which large synthetic libraries are prepared. Hundreds to thousands of compounds are able to be synthesized quickly and cost effectively if the reagents are plentiful, cheap, and able to take part in robust and straightforward reactions that are amenable to automation (Scheme 2.1).\(^{36,37}\)

Chirality and other forms of structural complexity are often imparted to chemical libraries either by using chiral or complex starting materials, which are generally more expensive than achiral building blocks, or by reactions involving expensive chiral catalysts which may be difficult to reclaim in an automated system. So in order to make thousands of compounds quickly and cost effectively, drug companies will avoid using more expensive materials, thus weeding out chirality and structural complexity before
Scheme 2.1: A simple combinatorial library. A simple starting material with three distinct functional moieties (1–3) can be modified with other simple building blocks giving rise to hundreds, if not thousands of unique chemical compounds. i) R₂OH, DEAD, PPh₃, THF ii) (CF₃CO)₂O iii) R₂OH, DEAD, PPh₃, THF – Then K₂CO₃, MeOH iv) R₃NH₂, DIEA, nBuOH – Then AcOH, H₂O, THF (3:1:1).

any compounds are even made, limiting the area of chemical space sampled by their compounds and consequently the possibility of finding new biologically active compounds. Not surprisingly, then is the fact that only one de novo* combinatorial compound has ever been FDA approved for use in the past 28 years (Figure 2.2).

Figure 2.2: Sorafenib. The only de novo combinatorial compound approved by the FDA, sorafenib is an antitumor drug.

* The drug, linezolid, although FDA approved in 2000 was synthesized as a result of the renewed development of an oxazolidinone project abandoned in 1987 and is therefore not considered a de novo compound.
Similarly, a review article published in 2001 summarizes the success of combinatorial synthesis to increase the activity of already discovered biologically active molecules; in a sense, simply performing structure activity relationship studies on the compounds in order to increase pharmacokinetic properties or to increase potency.\textsuperscript{39} Each of the libraries produced was a group of structural analogs to a pre-existing natural product and in most cases, library compounds were either not screened for activity or did not have better activity than their natural product precursors. Danishefsky stated, “A small collection of ‘smart’ compounds may be more valuable than a much larger hodgepodge collection mindlessly assembled. Thus, the decision on the part of several pharma companies to get out of the natural products business is gross foolishness.”\textsuperscript{40}

2.3 **Introducing Complexity into Synthetic Libraries**

The best way to incorporate structural complexity into compound libraries is to use a complex natural product or natural product-derived material in their syntheses. One of the most difficult problems to overcome when looking to natural products for drug design, however, is the lack of suitable chemical handles which are amenable to straightforward modification. Many of the functional groups appearing on natural products are alcohols and acids. This is ideal if there are unique, distinguishable sites for modification. However, natural products, especially macrolides, often will have several secondary alcohols in their structures and performing different reactions on each can prove very difficult and sometimes impossible (Figure 2.3).
Solid state combinatorial synthesis has shown some promise in the production of natural product analogs.\textsuperscript{39,41,42} However, a few drawbacks are inherent to a solid phase system:

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{erythromycin.png}
\caption{Erythromycin. The structures of erythromycin and its aglycone macrolide precursor, 6-deoxyerythronolide B.}
\end{figure}

1) The starting reagents must be anchored to the solid support, thus eliminating one chemical handle that is available for modification 2) There is not a universal solid support on which all reactions are able to be performed 3) As with solution phase syntheses, each step must be optimized, but at higher cost because of the cost of the solid support 5) A very large amount of solid support would have to be used in order to produce a large amount of compound. For these reasons, with very few exceptions, only small amounts of complex molecules have been synthesized in this manner. More commonly a mixture of both solution and solid phase syntheses is used. For example, Nicolaou synthesized a library of sarcodictyin analogs using both synthetic methods (Scheme 2.2). Sarcodictyin-containing derivatives are antitumor compounds that have a mechanism of action similar to the anticancer drug, taxol. Nicolaou’s syntheses of the sarcodictyin libraries require forty chemical steps and achieve only a 1\% overall yield.\textsuperscript{42}, 26
A 29-step solution-phase segment of the synthesis is employed in order to form the complex sarcodictyin core scaffold, which is then bound to a solid-phase support. The linked scaffold is then derivatized on the solid phase support affording chemical diversity and then the final compounds are detached.

Scheme 2.2: The synthesis of a sarcodictyin library. The library has a structurally complex core and three differentially modifiable chemical handles. The synthesis of the library has a solution phase section of 29 steps followed by another 11 steps for attachment to the solid phase and chemical derivatization. The overall yield of the synthesis is 1%.

Nicolaou’s synthesis of the sarcodictyin analogs shows the difficulty inherent in the incorporation of structural complexity and chemical diversity into chemical libraries. Though it is possible, many steps are necessary and yields are very low.
2.4 Introducing Complexity Using Molecular Biology

Methods in molecular biology have also been employed to increase the complexity of compound libraries. Most involve the engineering of a polyketide synthase (PKS) or other biochemical pathway in order to alter one single aspect of the natural product’s biosynthesis. One of the most studied and manipulated biosynthetic pathways is that of erythromycin and its aglycone precursor, 6-deoxyerythronolide B (6-dEB) (Figure 2.3). Early work in this area shows the versatility possible with combinatorial biosynthesis. Over 60 analogs of 6-dEB were produced through genetic manipulations of the 6-dEB PKS responsible for its production. The major drawback to this work was that the majority of the macrolides produced were only found in trace amounts and characterized by the agreement of mass spectrometry values with predicted structures. For this type of strategy to be used successfully for drug design, titers have to be increased significantly.

There are also several studies that have shown that diversification of the macrolide ring is possible through a combination of genetic manipulation and synthesis. The most successful involve genetic manipulation of the 6-dEB PKS followed by the addition to the fermentative culture of a synthetic analog of one of the natural precursors of the macrolide (Scheme 2.3). Unfortunately, only certain stereochemical configurations and certain sized precursors are incorporated into the pathway, severely limiting the number of compounds able to be made. Another related study involved producing five novel 6-dEB analogs in a genetically modified strain of *Streptomyces*
coelicolor and feeding these, in turn to a similarly modified strain of *Saccharopolyspora erythraea* to produce four erythromycin A analogs with a single R-group modification at carbon 13 and one 16-membered lactone analog (Scheme 2.4).\textsuperscript{44,47} This work has since been expanded to include several more similar 6-EB analogs in very small yields and more work is being done in the field to increase titer levels to make these compounds in more useful amounts.\textsuperscript{45,46} A remarkable amount of work has gone into these studies and to be fair, their main goal was only to show the possibilities afforded by combinatorial
biosynthesis. Even so, a large drawback to this type of bio-engineering is that it usually allows for only a few more compounds to be made with little or no possibility for the chemical addition or modification of functionality. Because of the drawbacks of each of these two main sources of drugs, other methods for drug discovery must still be sought.

Scheme 2.4: Erythromycin A analogs produced by non-natural 6-dEB analog feedings. N-acetyl cysteamine synthetic deritivates of natural precursors in the 6-deoxyerythronolide B biosynthetic pathway (first row) were incorporated into a mutant strain of Streptomyces coelicolor to produce the non-natural 6-dEB analogs (second row). Subsequent feeding of the 6-dEB analogs into a mutant strain of Saccharopolyspora erythraea yielded the corresponding methylated, hydroxylated and glycosylated Erythromycin A analogs (third row).
2.5 Nonactin and Nonactic Acid

In order to address the lack of structural complexity common to combinatorial libraries and still maintain the large number of compounds needed for a successful drug screening campaign, we set out to find an appropriate natural product starting material from which to make chemical libraries. Our main goal was to generate compound libraries from a single, easily modifiable, complex scaffold, therefore affording both stereochemical complexity and functional diversity in only a small number of synthetic steps. Conveniently enough, our lab has been interested for quite some time in the natural product, nonactin 1 (Figure 2.4).

![Nonactin and Nonactic Acid](image)

**Figure 2.4: Nonactin, higher homologs and nonactic acid monomer units.**

Nonactin 1 is a natural product produced by the actinomycete, *Streptomyces griseus*. It is a macrocyclic polyketide produced by a type II polyketide synthase. Nonactin is made up of two monomer units of each enantiomer of nonactic acid 2 arranged in an alternating (+)-(−)-(+)−(−) pattern around the macrocycle, which confers
S4 symmetry to the molecule, making it an achiral molecule made up of chiral building blocks.\textsuperscript{56, 57} The nonactic acid monomer units are ideal for the synthesis of natural product-like compound libraries. They are stereochemically complex, having four chiral centers. They are also able to be orthogonally modified, containing a carboxylic acid on C-1 and a secondary alcohol at C-8. The two different functional groups allow for easy manipulation and derivatization for the production of a large number of compounds based on the nonactic acid monomer unit. The monomer units\textsuperscript{*} are available as a racemic mixture of their methyl esters 3 in gram scale from an acid catalyzed methanolysis of pure nonactin.\textsuperscript{58, 59} The enantiomerically pure monomers are available through a series of bio-transformations mediated by *Rhodococcus erythropolis* (Scheme 2.5).\textsuperscript{58} Access to the individual enanantiomers is important for drug design as each enantiomer may exhibit different activity in vivo.\textsuperscript{60-62} Straightforward epimerization at C-2 and C-8 also allows for an additional layer of structural modification and gives rise to four stereoisomers in both the (+) and (−) enantiomeric series (Figure 2.5).

\textsuperscript{*}Hereafter, any nonactic acid or derivative thereof will be drawn as a single enantiomer to show proper relative stereochemistry. It should be noted, however, that most reactions were performed on a racemic mixture unless otherwise specified.
2.6 The Formation of a Triazoloester Compound Library from Methyl Nonactate Monomer Units

Armed with a stereochemically complex and diverse set of methyl nonactate monomer units (Figure 2.5), we were able to start designing natural product-derived scaffolds. Effective scaffolds are easily modifiable via one or two synthetic steps. Since 3 has both a secondary alcohol and a methyl ester as chemical handles, we were able to choose two separate means of chemical modification. We decided to employ the 1,3-
dipolar cycloaddition of alkynes and azides because it easily allows for the rapid generation of analogs containing a 1H-1,2,3-triazole. In order to incorporate the 1H-1,2,3-triazole into the library compounds, we converted the methyl ester to an azide and chose to derivatize with commercially available terminal alkynes. We also esterified the secondary alcohol at C-8 with commercially available acyl chlorides. From 8, our “triazoloester” compound library could be synthesized through chemical derivatization of the two chemical handles.

2.7 Synthesis of the Azidoalcohol Scaffold for the Triazoloester Library

We prepared the azidoalcohol scaffold 8 from the methyl nonactate 3 core (Scheme 2.6). The secondary alcohol was protected with a THP group and the ester reduced with LiAlH₄ to a primary alcohol which was then tosylation and displaced with azide. The secondary alcohol was then deprotected with tosic acid to afford the azidoalcohol precursor 8 to the library compounds. From here, a straightforward esterification with acyl chlorides and a Huisgen 1,3-dipolar cycloaddition to terminal alkynes in the presence of Cu(I) gave us our triazoloester library (Figure 2.6).

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* Some recent work has shown that analogs of the drug linezolid containing 1H-1,2,3-triazoles exhibited low micromolar-range, broad spectrum biological activity.⁶⁶
Scheme 2.6: Triazoloester Scheme. Library formation of the triazoloester-containing derivatives. The functionalizable azidoalcohol scaffold is highlighted.
Figure 2.6: Examples of compounds from the triazoloester library. A number of different chemical groups are able to be attached to a common scaffold.
2.8 Synthesis of a Triazoloamide Compound Library from Methyl Nonactate Monomer Units

Concurrently with the formation of the triazoloester library, Dr. Stephen Bergmeier’s lab synthesized another library based on the nonactic acid core to explore other functionality accessible from the same complex starting material (Scheme 2.7). For this library, an azidoacid scaffold was used instead of the azidoalcohol 8. They formed amides from the carboxylic acid at C-1, and again employed the 1,3-dipolar cycloaddition, but formed the azide on C-8 instead of C-1 as in the triazoloester library.

The secondary alcohol was tosylated and displaced by azide, causing an inversion of stereochemistry at C-8. The ester was then saponified to give the azidoacid scaffold 9. The acid was converted to the acyl chloride by oxalyl chloride and the amides were formed by the addition of primary and secondary amines. Finally, the azide was again derivatized with terminal alkynes to give the triazoloamide library (Figure 2.7).59
Scheme 2.7: Library formation of the triazoloamides. The azidoacid scaffold is highlighted. Note that C-8 stereochemistry is inverted from its natural configuration.
Figure 2.7: Examples of compounds from the triazoleamide library. A number of different chemical groups are able to be attached to a common scaffold.
By making these libraries, which will be discussed in this work, we have achieved our goal of making a large number of compounds in a combinatorial fashion by using a natural product-derived precursor, affording us both the structural complexity inherent to natural products, as well as the functional diversity and larger numbers of combinatorial libraries (Figure 2.8).

![Figure 2.8: Stereochemical Complexity and Functional Diversity. A picture of the triazoloesters and triazolooamides which shows elements of structural complexity afforded by the nonactin acid core (peach) and the functional diversity created with combinatorial synthesis (green).](image)

**2.9 Biological Activity**

All of the compounds from the two compound libraries were tested for antimicrobial activity via AlamarBlue assay. The AlamarBlue assay is a cell viability assay that uses the redox indicator, resazurin, to colorimetrically determine whether or not microorganisms are respiring (Figure 2.9). When the resazurin enters the cell and
becomes reduced to resorufin, it changes color from blue to pink. Several studies have indicated that this assay is a reliable indicator of cellular respiration and is highly reproducible.\textsuperscript{58-70} Combining the colorimetric change with a known concentration of compound being tested, the Minimum Inhibitory Concentration (MIC) of a biologically active compound can be inferred from the endpoint of the indicator. The MIC is defined as the minimum concentration of an antibiotic that is needed to visibly inhibit the growth of an organism after overnight incubation.\textsuperscript{71}

![Resazurin and resorufin](image)

**Figure 2.9:** Resazurin. The compound, resazurin is blue when oxidized. The natural respiration of the microorganisms reduces resazurin to resorufin, which is pink.

Lower MIC values correspond to more active or potent compounds. Using 96 well plates (Figure 2.10), this diagnostic was used to determine all of the MIC values listed in this work and all of the MIC values reported will be in units of micromolar concentration (\(\mu\text{M}\)). The glycopeptide antibiotic vancomycin, as a basis of comparison, has MIC values of 1 \(\mu\text{M}\) and 2 \(\mu\text{M}\) against *Staphylococcus aureus* and *Enterococcus faecalis*, respectively.

Another characteristic we were looking for was spectrum of activity, which is the number of different microorganisms against which the drug has activity. A drug that is only active against one bacterial species or group, Gram-negative for example, is said to
have a narrow spectrum of activity. A drug that is effective against both Gram-positive and Gram-negative as well as fungi would be said to have a broad spectrum of activity.

Figure 2.10: 96 Well Assay Pic. A serial dilution (Rows A-H, 1000 μM – 8 μM) of compounds to be tested (Columns 2-11) with appropriate positive (Column 12) and negative (Column 1) controls shown in a 96 well plate AlamarBlue assay. MIC values are recorded as the lowest concentration that a color change is visible to the eye. For example, the compound in column 6 shows a color change at row F and would have a corresponding MIC value of 32 μM.
CHAPTER 3

INITIAL TRIAZOLOESTER LIBRARY FORMATION AND DETERMINATION OF LEAD COMPOUNDS
3.1 Preliminary Triazoloester Library

Phillips synthesized a preliminary library of eleven triazoloesters (Figure 3.2) from five acyl chlorides and five terminal alkynes (Figure 3.1) for an initial volley of compounds to demonstrate our ability to make natural product-like compound libraries from the azidoalcohol scaffold 8. We found that both 30 and 31 had moderate MIC values (94 μM and 370 μM respectively) against Bacillus subtilis and 30 also had very low potency (1000 μM) against Staphylococcus aureus (Figure 3.3).

![Chemical Structures](image-url)

**Figure 3.1:** Chlorides and Alkynes used for preliminary Triazoloester Library. The 5 acyl chlorides and 5 terminal alkynes that were used in the synthesis of the preliminary triazoloester library.
Figure 3.2: Preliminary Triazoloester Library. The first eleven triazoloesters [20-31] made from five acyl chlorides [10-14] and five terminal alkynes [15-19].
3.2 Possible Mode of Action of the Lead Compound [30]

Upon observing that compounds 30 and 31 exhibited moderate activity against *B. subtilis*, Smith exposed the organism to 0.2 × MIC and 2 × MIC of 30 and viewed the results with a scanning electron microscope (Figure 3.4). A clear effect can be seen on the structure of the organism. It was initially thought that the compound was inhibiting or acting upon some aspect of the cell wall. We do know that the compounds have a bactericidal effect on *B. subtilis*, so the antibacterial effect must be severely detrimental to the organisms. Microarray RNA work performed later by Bolstad showed that several genes were either up- or down-regulated when exposed to 0.2 × MIC value of 30. Of particular interest was a putative cytoskeletal protein that was up-regulated. Cytoskeletal proteins are necessary for cell division and the doubling time for many species of bacteria is on the scale of hours. The incubation time of the *B. subtilis* with compound 30 was only one hour prior to the SEM imaging. If the mechanism of action of 30 was to inhibit some aspect of the cytoskeleton that hindered cell division; that could explain the structural deformities seen in the SEM images.

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* Unpublished Work
Figure 3.4: SEM images of *B. subtilis* exposed to [30] at 0.2 × and 2 × MIC value. A clear effect on the structure of the bacterium can be seen.

3.3 Structural and Stereochemical Importance of Methyl Nonactate for the Antimicrobial Activity of the Triazoloester Lead Compounds [30] and [31]

Other work by Phillips indicated that the stereochemistry and topology of the nonactic acid core material was necessary for the activity of the lead compounds. His work included synthesizing enantiomerically pure lead compounds 30 and 31 from (+) and (−) methyl nonactate 3 starting materials. Biological activity was retained only in the plus enantiomeric series. He also synthesized analogs based on a simpler, cyclohexane core scaffold 32-39. In addition to this, the cyclohexane analogs that were produced showed no activity save for that of the cis-(1,4) analog 32 of 30 which had an MIC of 125 µM against *B. subtilis* (Figure 3.5). This signifies that the nonactate-derived core scaffold is an important component of the triazoloester library and that this library has activity which can be optimized by varying the functional groups on the ester and the triazole ring.
Figure 3.5: Cyclohexane Analogs. Cyclohexane analogs produced by Phillips to determine the structural importance of the nonactic acid core of the triazoloesters.
CHAPTER 4

FIRST ROUND OF STRUCTURE ACTIVITY RELATIONSHIP STUDIES
4.1 Structure Activity Relationship Studies

Structure Activity Relationships (SAR) studies are used in order to determine which structural and chemical components of a drug are responsible for the compound’s biological activity. Structure Activity Relationships are determined through an iterative process that takes place over several rounds of chemical modification. Each round involves the systematic modification of one individual component followed by a biological assay. The compound with the desired biological activity is then chosen as the starting point for the modifications made in the next round. In this iterative, stair-step process, each round should yield a more potent compound than the last and an SAR profile can be formed which allows us to see trends in certain aspects of the chemical functionalities that are important for increasing antimicrobial activity. So for each round of SAR performed, more is learned about what makes our compounds effective as antimicrobials and the synthesis of the next round can be tailored accordingly.

Once it was evident that at least two out of the eleven triazoloester library compounds were biologically active, an SAR would be performed in order to determine in this particular case what functionality if any would be associated with lower MIC values and broader spectrum of activity. The only functionality we were concerned with changing at this early stage of the drug development process were chemical functionalities rather than any stereochemistry. With respect to the triazoloester library, that means altering the R-groups of both the ester and the triazole ring.
4.2 Structure Activity Relationship Studies Round 1

Scheme 4.1: The methyl 4-oxobutyrate azidoester [40] was functionalized by a series of 1,3-dipolar cycloadditions with propargylphenols

For this first Round of SAR, one of our lead compounds was picked as a starting point. Compound 31 was chosen as the template and the methyl 4-oxobutyric ester was held constant. A set of twelve propargylphenols 41-52 (Figure 4.1) prepared by Ewing were used for the Huisgen 1,3-dipolar cycloaddition reactions to alter the functionality of the triazolo R-group (Scheme 4.1).*

Figure 4.1: Propargylphenols. The propargylphenols prepared by Ewing used to make the compounds for the first round SAR.

* Unpublished Work
Starting with methyl nonactate 3, the methyl 4-oxobutyryl azidoester 40 was synthesized and the twelve propargylphenols were used to make the corresponding triazoloesters via Huisgen 1,3-dipolarycycloaddition in the presence of Cu(I) and ascorbate. All of the compounds were tested for antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus* and *Enterococcus faecalis*. No activity was found for any of the triazoloesters made in this first round (Table 4.1). In addition to the twelve compounds made that contained the methyl 4-oxobutyryl ester, three compounds were made that contained a phenyl ester and three of the propargylphenols (Scheme 4.2). The three phenyl triazoloesters also did not show any activity against the three microorganisms tested (Table 4.2).

![Scheme 4.2: The synthesis of compounds 66-68.](image-url)
Table 4.1: SAR Round 1a. Triazoloesters made from the methyl 4-oxobutyryl azidoester and assorted propargylophenols. *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis* were the organisms tested against. Dashes represent the lack of observable biological activity. None of the compounds were active.

<table>
<thead>
<tr>
<th>Alkyne #</th>
<th>Compound #</th>
<th>Compound Structure</th>
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<td></td>
<td></td>
<td><em>B. subtilis</em></td>
</tr>
<tr>
<td>41</td>
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<tr>
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</tr>
<tr>
<td>51</td>
<td>63</td>
<td><img src="image" alt="Compound Structure 11" /></td>
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<td>52</td>
<td>64</td>
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</table>

54
Table 4.2: SAR Round 1b. Triazoloesters made from the phenyl azidoester and three select propargyphenols. *Bacillus subtilis*, *Staphylococcus aureus* and *Enterococcus faecalis* were the organisms tested against. Dashes represent the lack of any observable biological activity.

<table>
<thead>
<tr>
<th>Alkyne #</th>
<th>Compound #</th>
<th>Compound Structure</th>
<th>MIC Values in µM Against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>B. subtilis</em></td>
</tr>
<tr>
<td>50</td>
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<tr>
<td>48</td>
<td>68</td>
<td><img src="image" alt="Compound Structure" /></td>
<td>-</td>
</tr>
</tbody>
</table>

4.3 Results and Discussion

Unfortunately, for this first round of SAR, none of the compounds made had any activity, but we were at least able to make some conclusions from that information. The methyl 4-oxobutyryl and phenyl esters used were most likely not responsible for conferring any activity to the original compounds 30 and 31. The propargyphenols also conferred no activity, which means that they were not an improvement over either the 2-pyridyl or the 3-acetanilide triazolo R-groups. From this information, we were able to tailor the next round of synthesis in an effort to maximize the probability for finding additional hits.
CHAPTER 5

SECOND ROUND OF STRUCTURE ACTIVITY RELATIONSHIP STUDIES
5.1 Structure Activity Relationship Studies Round 2

![Chemical structures](image)

**Scheme 5.1:** SAR Round 2. The pyridyl triazole R-group was kept constant and a series of acyl chlorides were used for esterification.

The compounds from the first round of SAR (Round 1) did not have any microbial activity. We concluded that neither ester from the original leads 30 and 31 was necessary for biological activity. The lack of the activity in Round 1 influenced this next round of SAR. The second round (Round 2) was designed to find a more potent compound by altering the R-group of the ester while keeping the triazole R-group constant *(Scheme 5.1)*.

For Round 2, the 2-pyridyl moiety seen in lead compound 30 was used as the constant triazole R-group. Eighteen acyl chlorides were then used to esterify the secondary alcohol at C-8 *(Figure 5.1)*. The 18 acyl chlorides were chosen based on their diverse structures and functionalities. Triazoloalcohol 69 was synthesized from the 1,3-dipolar cycloaddition of azidoalcohol 8 and 2-ethynyl pyridine. The 18 acyl chlorides were then attached to 69 at the secondary alcohol via esterification in pyridine *(Figure 5.2)*. The resulting triazoloesters for this round were then tested for antimicrobial activity *(Table 5.1)*.
Figure 5.1: SAR Round 2 Acyl Chlorides. These eighteen acyl chlorides [70-87] were used for the synthesis of the second round of compounds in the SAR studies.

5.2 Results and Discussion

The seven compounds that had activity showed significantly higher potencies than the compounds from Round 1 and the initial leads. Compound 91 contained a 1-(4-chlorophenyl) cyclopentyl ester and had significantly increased activity (4 μM) towards Bacillus subtilis. Compound 90, which contained an N-benzyl morpholino group, was not very potent towards B. subtilis (125 μM), but showed activity against both Staphylococcus aureus (250 μM) and Enterococcus faecalis (250 μM). This increase in activity indicates that the compounds can be functionalized to increase or broaden the spectrum of activity. The increased potency against B. subtilis exhibited by 91 is also encouraging for the further development of the triazoloester library. Compounds 103
and 105 also show low levels of activity (MIC = 500 µM) towards S. aureus. The information from this round of SAR indicates that overall, the 2-pyridyl group on the triazole ring is better than any of the propargylphenols from SAR Round 1. The data also indicates that bulkier groups used as the ester R-groups seem to afford lower MIC values.
Figure 5.2: SAR Round 2 Compounds Made. Triazoloesters made from the 2-pyridyl triazoloalcohol [69] and acyl chlorides [70-87].
Table 5.1: SAR Round 2 Assay Results. Triazoloesters made from the 2-pyridyl triazoloalcohol and various acyl chlorides. These seven SAR Round 2 compounds showed biological activity. *Bacillus subtilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes* and *Candida glabrata* were the organisms tested against. Dashes represent the lack of observable biological activity.

<table>
<thead>
<tr>
<th>Chloride #</th>
<th>Compound #</th>
<th>Compound Structure</th>
<th>B. subtilis</th>
<th>S. aureus</th>
<th>E. faecalis</th>
<th>C. glabrata</th>
<th>S. pyogenes</th>
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<td>-</td>
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<td>16</td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
5.3 The Triazoloamide Library

The triazoloamide library (Section 2.8) was being synthesized concurrently with the triazoloesters by the Bergmeier Group at Ohio University. Five amines [A – E] and 35 terminal alkynes 106-140 (Figure 5.3) were used to make a library of 161 compounds which were then tested for biological activity. For the sake of brevity, only the active compounds will be reported here (Table 5.2). The lead compounds from the preliminary triazoloamide library had comparable activities to the lead compounds of the preliminary triazoloester library (Chapter 3). Compound E-108 showed promise because of its broad spectrum of activity.
Figure 5.3: Amines [A-E] and alkynes [106-140] used to make the triazoloamide library.
Table 5.2: Preliminary Triazoloamide Library Assay Results 1/2: The 14 triazoloamides that had biological activity. Organisms tested against were *Bacillus anthracis*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cryptococcus neoformans* and *Saccharomyces cerevisiae*. Dashes represent the lack of any observable biological activity.

<table>
<thead>
<tr>
<th>Compound: [amine-alkyne]</th>
<th>Compound Structure</th>
<th>MIC Values in μM Against:</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>Gram-positive</td>
<td>Gram-negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>B. anthracis</em></td>
<td><em>B. cereus</em></td>
</tr>
<tr>
<td>B-107</td>
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<tr>
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<tr>
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<td>–</td>
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</tr>
<tr>
<td>C-133</td>
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### Table 5.2: Preliminary Triazoloamide Library Assay Results 2/2

<table>
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<tr>
<th>Compound: amine-alkyne</th>
<th>Compound Structure*</th>
<th>Gram-positive</th>
<th>Gram-negative</th>
<th>Yeasts/Fungi</th>
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</thead>
<tbody>
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<td></td>
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<td>B. anthracis</td>
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<td><strong>E-134</strong></td>
<td><img src="E-134" alt="Structure" /></td>
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<td>1000</td>
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</table>

* The stereochemistry of the furan ring was not reported.
The results of the preliminary triazoloamide library prompted an SAR study by the Bergmeier Group. One round of the triazoloamide SAR study gave rise to compounds 141 and 142 (Figure 5.4), each of which exhibited antimicrobial activity against B. subtilis (MIC = 64 µM), S. aureus (128 µM), and E. faecalis (128 µM).

5.4 Pharmacokinetic Studies on Lead Compounds 91, 141 and 142

The lead compounds from both the triazoloester and triazoloamide libraries had shown promising MIC values. Several of the library compounds also had a broad spectrum of activity. We decided that more work would inevitably produce compounds with antibacterial activity against more serious pathogenic organisms such as methicillin resistant Staphylococcus aureus (MRSA) and vancomycin resistant Enterococcus (VRE).

At this point in the drug development process, it is sensible to start determining the pharmacokinetic properties of our compounds.

Pharmacokinetics involves the absorption, distribution, metabolism, and excretion (ADME) of the compound in the body. In particular, we were curious about the absorption factors: oral bioavailability and half-lives of the compounds. Oral bioavailability is the amount of compound absorbed into the bloodstream after oral administration, reported as a percent of the total amount of drug ingested. The biological half-life of a compound is the amount of time it takes for a substance to lose half of its biological activity, potency, or to be half as effective. Pertaining to antibiotics, if the concentration of drug in the patient’s body drops below the MIC value, the drug becomes ineffective at killing infectious organisms. A good drug will be orally
bioavailable in order to be used in areas where patients may not have access to hospital care and have a high enough half-life to be functional, but not so high that it starts accumulating in tissues and/or causes damage.

We sent compounds 91, 141 and 142 to Ricerca Biosciences* for testing (Figure 5.4). Rats were dosed with one of the three compounds and each compound was administered both intravenously and orally. Experiments were performed in triplicate. Blood plasma samples were then analyzed for the presence of remaining compound at regular time intervals.

![Compounds 91, 141, and 142](image)

**Figure 5.4: Compounds sent to Ricerca for pharmacokinetic studies.**

### 5.5 Results and Discussion

The results summary that we were provided with indicated that compounds 141 and 142 had half-lives of 10 min and 23 min, respectively and neither was orally

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* Ricerca Biosciences is a company that offers a wide range of drug discovery and development services that are of great importance to smaller pharmaceutical companies and research groups that may not quite have the resources to do all of the preclinical tests on their own.
bioavailable. Compound 91, however had a half-life of 53 minutes* and had 7 % oral bioavailability. The longer half-life of ester 91 was surprising as esters are generally more susceptible to hydrolysis than amides. Perhaps the bulky ester side chain slows or prevents hydrolysis in vivo. Pharmacodynamic studies would have to be performed in order to explain the disparity between the two half-lives.

Pharmacodynamics relates to the mode of action of the drug in the body and how the drug is metabolized, revealing the major breakdown products of the drug. Knowing what the breakdown products are would allow for the design of a more stable or more effective drug.

Another possible explanation for the added stability of 91 is that both of the aryl rings present are substituted in some form, offering added protection against oxidation in vivo. Compounds 141 and 142 each contain phenyl rings in both the triazole and amide R-groups, both of which have accessible benzylic positions which are more easily oxidized than their phenyl ring counterparts in compound 91, which have quaternary carbons adjacent to the rings. Again, more experiments would have to be done to determine the reason for this discrepancy. A simple experiment would be to make analogs of 141 and 142 that had some substituent in the benzylic position on each, and then repeat the pharmacokinetic study. If the oxidation of the phenyl rings on the side chains is the reason for the short half-lives of 141 and 142, then each addition of a substituent would increase the half life.

* For comparison, the half-life of penicillin is about one hour.
An important thing to note is that 141 and 142, when compared to both 91 and methyl nonactate 3 have inverse stereochemistry at C-8. This could also have a detrimental effect on pharmacokinetics. To test this, analogs of 140 and 141 would have to be synthesized that have natural stereochemistry at C-8.

The next level of pharmacokinetic studies will be done later, once a more potent lead compound is found from the SAR studies. But, we do know at this early stage that the triazoloesters do in fact have some oral bioavailability. We can now concurrently determine the effects on potency and pharmacokinetics that any chemical changes have on our library compounds.
CHAPTER 6

THIRD ROUND OF STRUCTURE ACTIVITY
RELATIONSHIP STUDIES
6.1 Structure Activity Relationship Studies Round 3

Scheme 6.1: The formation of triazoloesters for Round 3 of SAR studies. Both ends of the azidoalcohol scaffold will be altered sequentially.

There were several questions brought about by the leads from Round 2 for each end of the compounds. For the ester: Is the cyclopentane ring important for activity? What happens if we change or remove the substituent on the phenyl ring? For the triazolo R-Group: If we add a substituent on the ring, will it increase or decrease activity? Is the pyridyl moiety important? What would happen if we went back and used the acetonilide group that conferred activity in 31? All of these questions could be answered with the synthesis and analysis of a simple array of compounds using several similar alkynes and acyl chlorides with significant subtle differences (Figure 6.1).

6.2 Topliss Scheme for Drug Design

In the 1970s, Topliss described a decision-making process wherein the synthesis and analysis of the drug-design process could be shortened by making fewer compounds. Though the process is shorter, useful information about the compounds can still be gathered. The Topliss scheme focuses on the fact that many substituent groups traditionally switched in drug design programs are essentially interchangeable until later on in the drug design process. Many drugs contain phenyl rings and many
drugs have substituents on these rings. In a traditional drug design scheme, a researcher may synthesize several compounds for a library with similar substituents: p-chloro, p-bromo, p-methyl, p-tert-butyl and o-methyl. The chloride and bromide have similar characteristics, as do the three alkyl substituents.

![Chemical structures](image)

**Figure 6.1: Building Blocks for SAR Round 3: Terminal alkynes [143-149] and acyl chlorides [150-153] used for the synthesis of SAR Round 3.**

Using the Topliss scheme, he could have reduced the number of compounds synthesized to two: the p-chloro and p-methyl, which would have saved time and resources (Figure 6.2). In order to determine the correct substituent, instead of haphazardly throwing on several random groups, the Topliss scheme calls for single, specific changes for each round of SAR. The changes and groups selected for each round encompass characteristics that contrast with one another. For example, chlorides are slightly electron rich and electronegative, whereas methyl groups are small and non-polar. Methoxy groups are electron rich and donating. More fine-tuning is possible in future steps, but to cut down on redundancy, each step contains the minimal number of compounds possible to determine the best course of action. The background selection
basis of this “decision tree” is rooted in the Hansch method of selection which relies heavily on mathematical formulas. Topliss took the idea, removed the tedious calculations and made it more accessible.

![Decision Tree Diagram](image)

**Figure 6.2: Topliss Scheme for Phenyl Substituents.** This tree summarizes which substituents should be synthesized and tested based on relative activity from the previous round of SAR. L = Less active, E = Equal activity, M = More active than the previous substituent. Using this scheme, if a 4-chloro substituent increases activity with respect to an unsubstituted phenyl ring, the next step would be to synthesize the compound containing a 3,4-dichloro substitution on the ring.

This scheme was used for the design of this round of SAR because of the questions we chose to ask. In determining the importance of the 4-chlorophenyl ring on the ester R-group, both the normal phenyl ring 152 and the 4-tolyl 151 groups were incorporated. According to the Topliss Scheme (**Figure 6.2**), this is the first decision to be made. We, however started with the chloride and needed to determine if perhaps
the original phenyl ring was more active, so we went both forward and backwards on
the scheme to answer our question.

In addition to the ester, elements of the Topliss scheme were applied to the aryl
group of the triazole R-groups. The most active compound 91 from Round 2 contained
the 2-pyridyl R-group. The plain phenyl ring was incorporated in this round as well as
the 4-chloro 144, 4-tolyl 146 and 4-nitro 149 groups. It was necessary to retain the 3-
acetamide 147 for proper comparison of the SAR of the ester groups and the 4-
acetamide 148 was included in order to determine the effect that the position of the
acetamide group had on activity. From these building blocks, a group of twenty
compounds was made (Figure 6.3) and tested for activity (Table 6.1).

6.3 Synthesis of the SAR Round 3 Compounds

The compounds were synthesized from the esterification of azidoalcohol 8 with
chlorides 150-153 to give the azidoesters 154-157 (Scheme 6.2). The azidoesters were
then modified via the 1,3-dipolarycycloaddition with terminal alkynes 143-149 in the
presence of Cu(I) to give the Round 3 triazoloesters 158-177 (Figure 6.3). One
compound, 173, proved difficult to make in this fashion, so it was made by first
performing the 1,3-dipolarycycloaddtion with 2-ethynyl pyridine, giving triazoloalcohol
69, which was then esterified with chloride 153 (Scheme 6.3). The esters in this round
were also synthesized using activated zinc and benzene instead of pyridine in order to
afford a more straightforward purification procedure.77
Scheme 6.2: Synthesis of the azidoesters for SAR Round 3.

Scheme 6.3: Synthesis of 173.
Figure 6.3: SAR Round 3 Compounds. The twenty triazoleesters that were made for Round 3.
Table 6.1: SAR Round 3 Assay results (2/2): Twelve triazoloesters from SAR Round 3 that showed biological activity. *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Candida glabrata* *Streptococcus pyogenes* and *Escherichia coli* were the organisms tested against. None of the compounds showed activity against *S. pyogenes*, therefore the column has been excluded from the table. Dashes represent the lack of observable biological activity.

<table>
<thead>
<tr>
<th>Chloride #</th>
<th>Alkyne #</th>
<th>Compound #</th>
<th>Compound Structure</th>
<th>B. subtilis</th>
<th>S. aureus</th>
<th>E. faecalis</th>
<th>C. glabrata</th>
<th>E. coli</th>
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<tbody>
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<td>147</td>
<td>170</td>
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<td>153</td>
<td>143</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
6.4 Results and Discussion*

All of the compounds tested that had activity showed some activity against *B. subtilis*. The changes in activity for this round will focus on the subtle changes in these numbers. The compounds in Row A, all containing the 1-(4-chlorophenyl)cyclopentyl ester, were the first few tested in the biological assay (Table 6.2). Compounds 158, 159 and 160 did not show any activity and as a result, several of the compounds in Columns 2, 3, and 4 were not made because it was unlikely that any of these would have more activity than their parent compounds in Row A. The fact that those three compounds did not show antimicrobial activity indicated that “greasy” substituents on the aryl group of the triazole ring do not confer activity and in fact reduce it. Some of the nitro-containing compounds in Column 7 proved difficult to synthesize and therefore only two were able to be tested.

* For the following discussion on R-group trends, refer to Table 6.2

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Table 6.2: SAR Round 3 Grid. Grid representation of the combination of compounds made for Round 3. Compounds that were synthesized are labeled with their numbers. MIC values are given in µM for compounds with activity. Compounds with no activity are labeled in red.

<table>
<thead>
<tr>
<th></th>
<th>Column 1</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4</th>
<th>Column 5</th>
<th>Column 6</th>
<th>Column 7</th>
</tr>
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<tbody>
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<td>158</td>
<td>159</td>
<td>160</td>
<td>161</td>
<td>162</td>
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</tr>
<tr>
<td></td>
<td>B. subtilis – 4</td>
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<td></td>
<td></td>
<td>B. subtilis – 4</td>
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</tr>
<tr>
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<td>S. aureus – 500</td>
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<td>S. aureus – 1000</td>
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<tr>
<td>163</td>
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<td></td>
<td>B. subtilis – 4</td>
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<tr>
<td></td>
<td>E. faecalis – 1000</td>
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<td>B. subtilis – 4</td>
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<td>B. subtilis – 16</td>
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<td>E. coli – 125</td>
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<tr>
<td></td>
<td>E. coli – 1000</td>
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<tr>
<td>173</td>
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<td>177</td>
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</tr>
<tr>
<td></td>
<td>B. subtilis – 31</td>
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<td>B. subtilis – 16</td>
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<tr>
<td></td>
<td>S. aureus – 1000</td>
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<td>B. subtilis – 8</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>B. subtilis – 16</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

An important trend was observed in the activity of all of the compounds made in Columns 1, 5 and 6. The acetamide and pyridyl moieties seem to be very important for conferring activity in this library and the 4-acetamide seems to be slightly more active than the 3-acetamide. We conclude from this trend that an important characteristic of the triazolo R-group is to be electron-rich and able to donate electron density to its target, perhaps for hydrogen-bonding. This idea is supported by the fact that the 4-nitro compounds (Column 7) showed no activity, probably due to the fact that the nitro groups are less electron-donating than the acetamides or pyridyl moieties.

The last trend observed was that compounds in Row D had reduced activity towards B. subtilis. These compounds all lacked the cyclopentane ring on the ester R-
group. The lack of the ring reduces rigidity of the molecule, possibly impairing any binding specificity afforded by the parent ester group (Row A).

6.5 Future Work for SAR Round 3 Compounds

If the characteristics of this group of compounds were to be further developed to increase activity, future work would include slightly increasing the mass of the ester side chain; making a 3,4-dichlorophenyl derivative would be the very next step. Another plan for this round originally was to substitute a cyclohexane ring in place of the cyclopentane to determine whether or not increasing the size of the ring would have an effect on activity as well. These compounds were not synthesized, but they could be another step towards increasing activity for this group of compounds.

Because of the electron-donating characteristics of the most effective triazolo R-groups, it could be assumed that other groups such as amines, alcohols and acids could also confer activity. To determine the validity of that hypothesis, synthesis and comparison of analogs of 91, 161 and 162 that contain electron-donating, para-substituents would be done.

At this point in the drug discovery process, it would also be valuable to ascertain the target of these compounds in order to learn about the mechanism of action. That information would be helpful in making the next determination for what will make this library of compounds more active.
6.6 Enantiomerically Pure Lead Compounds

Because three compounds with low MIC values were found: 91, 161 and 162, it was then necessary to determine which enantiomeric series, if any, retained activity. Phillips had previously determined that only the enantiomers of both 30 and 31 derived from methyl (+)-nonactate retained activity. Because these compounds are based on the same core scaffold 8, we expect these new leads to retain the same characteristics.

The enantiomerically pure compounds from Round 3 were synthesized in the same manner as their racemic counterparts, however enantiomerically pure methyl (+) and (−)-nonactate 3 were used as starting materials (Figure 6.4).

Figure 6.4: Enantiomerically Pure Lead Compounds from Round 3. The compounds on the left are made from the minus enantiomer of methyl nonactate and those on the right are made from the plus enantiomer.

Previously, the plus enantiomers were twice as potent as their minus counterparts. The compounds in this study all exhibited low MIC values, compounds 190, 192, 193 and 194.
each had MIC values of 4 μM. Compound 191 had a value of 2 μM and 195 had a value of 8 μM. These results were puzzling and require further study to be understood completely. Perhaps there was some contamination interfering with 195.

6.7 Amide Substitutions of the Lead Compound Esters:

![Scheme 6.4: Synthesis of the reverse triazoloamides.](image)

To determine the differences between the triazoloesters and triazoloamides, three reverse triazoloamide* analogs of the lead triazoloesters, 91, 161 and 162, were synthesized. The previous, pharmacokinetic comparison of triazoloester 91 and the triazoloamides 141 and 142 in Chapter 5.4 was not a direct comparison of esters and amides due to the fact that the R-groups on all three compounds compared were different. An appropriate comparison would require all stereochemistry as well as

* The term “reverse triazoloamides” acknowledges that the term “triazoloamides” refers to the compounds made by the Bergmeier lab discussed in Chapter 5.3. The triazoloamides have the triazole ring and amide at the C-8 and C-1 (of methyl nonactate), respectively; the reverse triazoloamides have the opposite connectivity.
functionality to be the same except for the identity of the ester/amide functionality (Table 6.3).

The reverse triazoloamides were synthesized from methyl nonactate 3. An $S_N2$ displacement of the alcohol by triphenylphosphine dibromide gave the methyl 8-epi bromo nonactate 196. The ester was then reduced to the primary alcohol 197 and the bromide was displaced with azide 198. The azidoalcohol was then reduced with palladium on carbon under a hydrogen atmosphere to give the diversifiable aminoalcohol 199. The inverted stereochemistry at C-8 of the bromide 196 allowed for the aminoalcohols and the resultant reverse triazoloamides to have natural stereochemistry (relative to methyl nonactate 3) because of the double $S_N2$ displacement. The amine was then selectively acylated with chloride 150 to afford the amidoalcohol 200. The primary alcohol was then tosylated and displaced by azide. A 1,3-dipolar cycloaddition was then performed with the appropriate terminal alkynes: 143, 147 and 148 in the presence of Cu(I) to afford the reverse triazoloamide analogs of 91, 161 and 162 (Scheme 6.4).

The data showed that the amides were half as potent as the esters against B. subtilis (Table 6.3). Although a good comparison would also contain pharmacokinetic data, the compounds have not yet been tested. Perhaps the mode of action of these compounds is augmented by the ester functionality in particular.
Table 6.3: Assay Data for the Reverse Triazoloamides. The three lead triazoloesters [91, 161 and 162] (Left) and the corresponding reverse triazoloamides [204-206] (Right). Below the structures are the MIC values against *Bacillus subtilis*.

<table>
<thead>
<tr>
<th>Triazoloester Leads</th>
<th>MIC Value</th>
<th>Reverse Triazoloamides</th>
<th>MIC Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>4 μM</td>
<td>204</td>
<td>8 μM</td>
</tr>
<tr>
<td>161</td>
<td>4 μM</td>
<td>205</td>
<td>8 μM</td>
</tr>
<tr>
<td>162</td>
<td>2 μM</td>
<td>206</td>
<td>8 μM</td>
</tr>
</tbody>
</table>

MIC Values against *B. subtilis*
CHAPTER 7

FOURTH ROUND OF STRUCTURE ACTIVITY RELATIONSHIP STUDIES
7.1 Structure Activity Relationship Studies Round 4

One final group of compounds was synthesized for the triazoloester library. Two of these compounds, 210 and 211, were made in order to determine the importance of the placement of the nitrogen in the pyridine ring. Compound 201 was isolated during the purification of amidoalcohol 200 as a di-addition side product. Compound 212 was made in order to see if the inclusion of the 4-phenyl piperidyl R-group off of the triazole would confer more antimicrobial activity. That particular R-group was chosen because it was a component of several promising compounds from a later round of SAR of the triazoloamide library (Figure 7.1). Each of these compounds was tested for antimicrobial activity (Table 7.1).

![Figure 7.1: SAR Round 4 Compounds. These 4 compounds were synthesized for SAR Round 4](image-url)
Table 7.1: SAR Round 4 Assay Data. *Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Candida glabrata* and *Streptococcus pyogenes* were the organisms tested against. Dashes represent the lack of any observable biological activity. Compound [91] is included for comparison.

<table>
<thead>
<tr>
<th>Compound #</th>
<th>Compound Structure</th>
<th>B. subtilis</th>
<th>S. aureus</th>
<th>E. faecalis</th>
<th>C. glabrata</th>
<th>S. pyogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td><img src="image1" alt="Compound 91" /></td>
<td>4</td>
<td>500</td>
<td>500</td>
<td>–</td>
<td>N.D.</td>
</tr>
<tr>
<td>210</td>
<td><img src="image2" alt="Compound 210" /></td>
<td>2</td>
<td>1000</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>211</td>
<td><img src="image3" alt="Compound 211" /></td>
<td>2</td>
<td>1000</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>201</td>
<td><img src="image4" alt="Compound 201" /></td>
<td>64</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>212</td>
<td><img src="image5" alt="Compound 212" /></td>
<td>1</td>
<td>64</td>
<td>250</td>
<td>–</td>
<td>8</td>
</tr>
</tbody>
</table>
7.2 Results and Discussion

Compounds 210 and 211, when compared to the lead compound 91 (containing the 2-pyridyl R-group), each retain activity against both B. subtilis and S. aureus. However, while potency against B. subtilis was increased from 4 μM to 2 μM, it was decreased against S. aureus from 500 μM to 1000 μM, and the activity against E. faecalis was lost completely. This indicates that the 2-position of the nitrogen in the pyridyl ring is necessary for retaining activity against the more important pathogens. Compound 201 only had moderate activity (64 μM) against B. subtilis. This compound does not have a triazole ring, which is likely an important feature for the activity of the triazoloester library. One way to determine the importance of the triazole ring would be to synthesize several analogs with the same R-groups but without the triazole ring (Figure 7.2). The side chain would have to be approximately the same length as the side chain on the working compounds. The increased saturation of the side chains would make the proposed compounds more “floppy,” reducing some of the rigidity provided by the natural product-derived precursor, which may decrease activity.

Figure 7.2: Triazole Ring Importance. These compounds could be synthesized in order to determine the importance of the triazole ring in the triazoloester and triazoloamide libraries.
The final compound in this round was 212, which gained an increased spectrum of activity against both *E. faecalis* and *S. pyogenes* and had the highest potency against *S. aureus* of any of the triazoloesters to date. This increase in activity is unquestionably due to the inclusion of the 4-phenyl-piperidine as the triazolo R-group.

### 7.3 Structure Activity Relationship Conclusions

A library of triazoloesters was synthesized and tested for biological activity over several rounds of SAR. The library was based on a natural product-derived precursor. The initial leads 30 and 31 were used and referenced as starting points for diversification.

For Round 1, the methyl oxobutyryl ester was held constant and several propargyl phenols were used for chemical diversification of the triazole ring. None from the first round was active.

For Round 2, the 2-pyridyl triazolo R-group was kept constant and the esters were changed. Round 2 yielded 7 compounds that showed biological activity. One triazoloester, 91, from Round 2 contained a 2-pyridyl R-group off of the triazole and a 1-(4-Chlorophenyl)cyclopentane* as the R-group on the ester. Compound 91 had the highest potency of Round 2 and was used as the starting point for Round 3.

Nineteen compounds were synthesized for Round 3, for which a Topliss scheme was employed for the R-groups on both ends of the molecule. Round 3 was the source of much data with respect to the SAR. The pyridyl ring and acetamides show the most

*This ester R-group was the best ester and used through the subsequent rounds of SAR.*
potent and broadest spectrum of activity when used as the triazolo R-groups. The ester needs to be bulky, with the 1-(4-chlorophenyl)-1-cyclopentyl ester retaining the highest activity overall.

It was found that the plus enantiomers of the compounds exhibit the highest potency, but there is still a comparable amount found in the minus series. When the three best esters were replaced with their corresponding amides, the esters were universally twice as potent.

When moving the pyridine nitrogen around the ring, we found that maintaining the nitrogen in the 2- position afforded the broadest spectrum and the highest potency against *B. subtilis*, *S. aureus* and *E. faecalis*. The triazole ring seems to be essential for the activity of this library due to the considerable decrease in activity from the accidental di-acetylated compound 201. Finally, the 4-phenyl piperidine group, when used as the R-group on the triazole ring conferred both a broad spectrum of activity and low MIC values.

The investigation on the toxicity of these compounds is currently underway. Preliminary data collected by Martin suggests that the more biologically active triazoloesters (compounds 91, 161, and 162) are non-toxic to mammalian cells.‡

The overall SAR profile that we begin to see for these compounds indicates what is important for their activity as well as the structural and chemical requirements necessary to interact with their biological target(s). The bulkiness and rigidity of the 1-(4-chlorophenyl)-1-cyclopentyl ester likely has strong interactions with a hydrophobic

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* Each of these organisms is Gram-positive.
† Unpublished Work
pocket in an enzyme target. The eudismic ratio of the (+) to the (−) enantiomeric series indicates that there is some stereochemical restriction to the binding site of the biological target.

The triazole ring likely offers some sort of electrochemical benefit first described by Genin but not entirely understood. The nitrogen of the triazole R-group seems to be important for increasing the spectrum of activity to both S. aureus and E. faecalis. Indeed, compounds Compounds 91 and 212 both contain a nitrogen in the same location relative to the triazole ring (Figure 7.3). This nitrogen would be able to take part in hydrogen bonds. The pyridyl nitrogen (pKa ≈ 5) would be deprotonated at physiological pH and the piperidine nitrogen (pKa ≈ 9) would be protonated, allowing the former to act as a hydrogen bond acceptor and the latter as a donor. This means that they would likely interact with either the peptide backbone of

\[ \text{Figure 7.3: Alignment of the nitrogen position in compounds [91] and [212]} \]

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*Genin mentioned that the overall electronics of the triazole ring in linezolid analogs were important for the activity of the compounds. He suggested that the triazole ring could play a role in partitioning the compounds through the bacterial cell wall.*
an enzyme or perhaps polar side chains such as the acids of aspartate or glutamate. More work will have to be done concerning the biological target to ascertain the actual role and importance of the nitrogen in that position.

There is still not enough data to determine the importance of the triazole R-group beyond the nitrogen because of the large differences among the groups that still confer activity. The 3- and 4- acetamides don’t confer much activity towards *S. aureus* or *E. faecalis*, but still maintain their potency against *B. subtilis*, which indicates perhaps the target may have a slightly different binding pocket in each species or perhaps even a completely different target from species to species. There may also be another hydrophobic interaction between the 4-phenyl-piperidine group and the target that may be exploited further.

Overall, there seems to be a target that is satisfied by the stereochemical structure of the nonactate core containing two groups that can engage in hydrophobic interactions at either end of our molecules, some sort of electronic interaction with the triazole, and a nitrogen located one methylene carbon away from the triazole ring that may be taking part in a hydrogen bond or salt bridge interaction.

### 7.4 Future Work

For the continued work on this library project, several avenues are open to further study. One of which, suggested in Section 7.2, concerned the validation of the importance of the triazole ring. Another set of experiments would focus on increasing
the bulk of the ester R-group, either by increasing the ring size from five carbons to six or adding another chlorine on the 3- position of the phenyl ring.

The next step for the triazolo R-group should be to apply the Topliss scheme to the phenyl ring on the piperidine moiety exhibited in compound 212. Adding a chloro, methyl, and methoxy group to the 4- position would be the very next step.

Another problem that should be investigated is the pharmacokinetic differences between the amide analogs 204, 205, and 206 of the lead compounds 91, 161, and 162 in order to get a better idea of the factors that influence the drugability of these compounds.

The triazoloester library has the potential to yield an effective new antibiotic. Though much more work is necessary for the full development of an FDA approved drug, the fundamental requirements are already present. The most potent library compound, 212, has MIC values within an order of magnitude to those of vancomycin against Staphylococcus aureus, Enterococcus faecalis and Streptococcus pyogenes. If the triazoloester library can continue to be developed with lower MIC values while still remaining non-toxic and orally bioavailable, a new chemical entity with antimicrobial activity could be approved by the Food and Drug Administration in the near future.
CHAPTER 8

SPINOSYN TO SPINOSENE

Bioengineering a Natural Product Scaffold
8.1 Spinosyn to Spinosene: Bioengineering a Natural Product Scaffold

Our first attempt to make a complex new compound library was to reprogram a polyketide synthase (PKS) of a macrolide producer, *Saccharopolyspora spinosa*. Similarly to *Saccharopolyspora erythraea* and the production of erythromycin, *S. spinosa* makes a group of macrolides called the spinosyns.⁷⁸,⁷⁹ The spinosyns are a group of potent insecticides that are non-toxic to humans.⁷⁹-⁸² Spinosyn A is the most abundant of the spinosyns and was the subject of our studies. Using the same approach as taken by McDaniel et al., we were going to reprogram the PKS to make non-natural analogs of the natural product.⁵³ In contrast to McDaniel’s work (Section 2.4), our goal was to make only one analog that was amenable to straightforward chemical modification in order to accomplish our overall goal of making large libraries of natural product-like chemical compounds.
8.2 Fatty Acid Synthases

Macrolides and polyketides are all produced in a similar fashion by polyketide synthases (PKSs), which function similarly to fatty acid synthases (FASs). Fatty acid synthesis occurs as an iterative process of condensations and reductions by a collection of enzymes either as a single megacomplex (type I) or as discrete but dependent enzymatic units (type II). Prokaryotes generally employ type II fatty acid synthases. Fatty acid synthesis starts with the condensation of an acetate unit from acetyl coenzyme A (acetyl-CoA) with a malonate unit from malonyl-CoA orchestrated by three enzymatic reactions mediated by three enzymes in the elongation process: an acyl carrier protein (ACP), acyl transferase (AT) and a keto synthase (KS) (Figure 8.1). The growing chain is then 2 carbons longer with a β-ketone that needs to be reduced, which is accomplished by the actions of a keto synthase (KS), a dehydratase (DH) and an enoyl reductase (ER). The KR reduces the nascent ketone of the extended chain to an alcohol using NADPH as a cofactor. The alcohol is then eliminated by the action of the DH domain, releasing water. Finally, the resultant double bond is reduced by the ER domain using cofactor FADH and another molecule of NADPH. These reduction or ‘tailoring’ steps result in a methylene carbon in place of the β-ketone. The overall action of all six enzymes results in a saturated fatty acid chain that is two carbons longer than the nascent chain. This growing chain is then transferred back to the KS domain and the process repeats until the chain grows to the desired length when it is then released from the ACP by the hydrolytic action of a thioesterase (TE) domain.
8.3 Polyketide Synthases

Polyketide synthases differ from fatty acid synthases in three main ways: 1) there are more choices for starter units than the simple acetyl group used by FASs (Figure 8.2) 2) extender units may be acetyl, malonyl or methylmalonyl among others (dependent upon specificity of the AT domain) and 3) the process is not iterative and not all of the tailoring enzymatic domains are present for each cycle of elongation.\textsuperscript{1,83,85,86} These three differences, along with some post PKS modifications (oxygenations, glycosylations and methylations) are responsible for the large amount of diversity seen in polyketides when compared to fatty acids (Figure 8.3).
Figure 8.1: Fatty Acid Synthase Enzyme Mechanisms. The first extension, reduction and the second extension steps of a fatty acid synthase. KS: Keto Synthase, ACP: Acyl Carrier Protein, AT: Acyl Transferase, KR: Keto Reductase, DH: Dehydratase, ER: Enoyl Reductase, CoA: CoEnzyme A. A) The loading of the KS domain with acetate from acetyl-CoA B) Loading of ACP domain with malonate from malonyl-CoA mediated by the AT domain C) Extension step, decarboxylation and condensation of the nascent chain with a malonyl extender unit D) Reduction steps mediated by KR, DH, and ER domains to fully reduce the ketone to a saturated carbon E) Transfer of extended, reduced chain to KS domain to prepare for the next round of extension F) Second extension.
Figure 8.2: The common building blocks used in the synthesis of polyketides.

The group of enzymatic domains that are responsible for each round of extension and tailoring in a type I PKS are known collectively as a module. Each module is only used once for each molecule produced and determines the oxidation state of each added extender unit. Several modules are strung together to form a full type I PKS and more than one module may be encoded by a single gene. The final module will have a TE domain to cleave the polyketide chain from the final ACP, concurrently cyclizing when appropriate. After the polyketide has been formed by the PKS, further processing and modifications occur in order to complete the secondary metabolite being produced.
Figure 8.3: Fatty acids vs. Polyketides: The top six compounds are all examples of fatty acids found in nature. The bottom five compounds are all natural products made by polyketide synthases. Even though the same enzymatic domains are used for both fatty acid synthesis and polyketide synthesis, much more diversity is seen in the structures of the polyketides.
8.4 Polyketide Synthase Modifications

Extensive studies into how PKSs were organized and functioned allowed for genetic studies and manipulations to be performed on the biosynthetic pathways of many macrolide and polyketide antibiotics. This began in 1991 when Donadio et al. proposed and described the similarities of the enzymatic domains of the PKS that encodes 6-dEB to those of FASs. They also described the connection between linear organization of the encoded PKS with the resultant products of each extension (Figure 8.4) and observed that there is some leeway regarding substrate specificity for each domain, suggesting that altering one domain through genetic modification would result in the production of a “tailored” polyketide. Two years later, they described the reprogramming of the same PKS that resulted in the production of an erythromycin analog (Figure 8.4). According to the PKS model that they previously proposed (3 genes encoding 6 rounds of extension), the inactivation of the ER domain would result in the biosynthesis of an analog of erythromycin that was not fully reduced after its fourth round of elongation. They identified the genomic sequence that encoded the unique ER domain of the fourth module in the 6-dEB PKS, which is responsible for the fourth round of extension in the biosynthesis of erythromycin. Using sequence homology to known, corresponding FAS domains, they located the sequence encoding the NADPH binding site of the ER domain. By altering four bases in the genome, they were able to effect sufficient changes to prevent the necessary cofactor from binding and inactivate the ER domain without introducing damaging structural changes to the rest of the enzyme complex. This resulted in the formation of a fully completed
erythromycin analog that was left unsaturated at one targeted site (Figure 8.5). This analog had 200–500 fold less antibiotic activity than the natural product, indicating that very small changes in chemical structure have a significant effect on biological activity.
Figure 8.4: 6-dEB PKS. A graphical representation of the PKS that encodes the biosynthesis of the 6-dEB precursor to erythromycin. Top: the wild type PKS, Bottom: ER inactivated PKS resulting in an unreduced fourth round of elongation.
The work by Donadio ignited research into domain inactivation, swapping and addition in and from many different PKSs from a variety of organisms. Some of the other structural changes possible include the increase and decrease of macrolide ring size, side chain modifications, starter unit modification, and extender unit modification by the swapping of AT domains.\textsuperscript{52}

![Chemical structures](image-url)

**Figure 8.5: Anhydroerythromycin.** The 6-DEB polyketides (top) and resultant erythromycins from post-PKS processing (bottom). $\Delta^{6,7}$-anhydrooxyerythronolide B is the resultant polyketide from the reprogramming of the 6-DEB PKS that results in the formation of $\Delta^{6,7}$-anhydroerythromycin C.
8.5 Bioengineering of the Spinosyn Biosynthetic Pathway

Scheme 8.2: Spinosyn to spinosene.

The precedent of the successful inactivation of the ER domain of the 6-dEB PKS encouraged us to do the same with spinosyn A. Although the spinosyns are potent insecticides, even small changes in structure can have a significant effect on biological activity. It is therefore possible that changes in structure may also change the mode of biological activity, perhaps affecting bacteria instead of insects.

A large problem with the genetic manipulation of PKSs is that although new, non-natural products are made, a significant amount of product yield is lost due to diminished production from the producing organism. For the large scale production of the sixty bioengineered analogs of 6-dEB produced by McDaniel et al. in 1999, the growth and maximum yield conditions for each strain would have to be optimized, which would involve a significant amount of work. Our solution for this problem was to only make one particular non-natural product instead of making many, reducing the amount of strain improvement necessary for increased production.
There have already been studies done relating to the optimization of spinosyn production from *Saccharopolyspora spinosa*. Our increased numbers for library formation would be afforded by the subsequent chemical modification of the engineered non-natural analog.

Spinosyn is biosynthesized by a PKS encoded by 5 genes (*spnA*-*spnE*) responsible for 10 rounds of extension (*Figure 8.6*). The overall goal of the genetic engineering of this project was to inactivate the unique ER domain in module 2 of the spinosyn PKS in a similar manner by which Donadio altered the 6-dEB PKS in 1993.\(^90\)

Two adjacent glycine residues in the NADPH binding site would be changed to a serine and a proline. This structural change in the enzymatic domain proved to be enough to inactivate the ER domain of the 6-dEB PKS and should also prove effective in the spinosyn PKS as many of the PKS domains are highly conserved from species to species.\(^90,91\) The protein sequence HSAA\text{GG}VGMA is the conserved NADPH binding site in SpnB. The genetic sequence 5′ – CATTCGGCCGCC\text{GGTGGG}GTTGGGATGGCC – 3′ (the two glycine residues are highlighted in red) is the corresponding sequence in the *spnB* gene. In order to effect the necessary changes in the protein sequence, the genetic sequence must be altered. This is done through two successive rounds of homologous recombination, which is a natural mechanism by which genetic material is swapped between organisms.\(^27\) Similar DNA sequences align and swap material to foster genetic diversity. It is possible to intervene in this genetic swapping through the use of plasmids. By cloning a gene into a plasmid and making small changes we are then able
to re-introduce specific modifications into the gene, reintroduce the plasmid into the host organism and, if a homologous recombination occurs, alter the genome of that organism. Regions of homology are necessary on each side of the segment of altered DNA, the longer the homologous sequences, the more efficient the recombination events are. Since the targeted gene in this work is spnB, we will be using the entire gene to incorporate regions of homology.

Our plan was to clone the spnB gene into a plasmid, perform a PCR-based site directed mutagenesis on the sequence above to change GGTGGG into AGTCCA which encodes for serine (AGT) and proline (CCA) respectively, then reintroduce the plasmid into the parent bacteria, S. spinosa, which would then cause the altered DNA to be taken up by the bacterial chromosome. An extra round of recombination would be necessary, however, because there would be no selectivity between each step of recombination unless there was some defining phenotypic characteristic between the wild-type parent strain and the mutant “spinosene” producer. Some sort of screening would be necessary. We decided to employ two markers in our design: hygromycin resistance and sucrose sensitivity. By the first homologous recombination of the wild-type chromosomal DNA with the first plasmid containing a hygromycin resistance cassette (hygR) and sucrose sensitivity marker (sacB) in place of a majority of the spnB gene, any resulting mutants would have resistance to hygromycin and be unable to grow on media containing sucrose. The second cross would re-introduce a functional spnB gene with the exception of the ER domain containing the non-functional
Figure 8.6: Spinosyn PKS. A graphical representation of the PKS that encodes the biosynthesis of the aglycone precursor to spinosyn. AT: acyl transferase, ACP: acyl carrier protein, KS: keto synthase, KR: keto reductase, DH: dehydratase, ER: enoyl reductase, TE: thioesterase. Note that the ER domain of the spnB gene, encoding for the second extender module is inactive, leaving the double bond (shown in red) unreduced for that round of elongation and for the rest of the biosynthetic pathway.
mutation in the NADPH binding site into the mutant *S. spinosa* chromosome. This mutant would have sensitivity to hygromycin, but would be able to grow in the presence of sucrose. Also, the resultant mutant would conceivably produce the non-natural product spinosene. Overall, 2 plasmids need to be made in order to successfully replace the ER domain with the inactivated copy: First, a plasmid with a truncated *spnB* gene with regions of homology on either side of a hyg<sup>R</sup> sacB cassette, and second, a plasmid with a copy of *spnB* containing an altered sequence at the NADPH binding site of the ER domain (Figure 8.7).

**Figure 8.7:** Plasmids necessary for the two rounds of homologous recombination. These two plasmids would be responsible for the two homologous recombination events leading to the final mutant *S. spinosa* that would produce the non-natural product, “spinosene”. Colored bars represent endonuclease restriction sites for use in the design and construction of the plasmids.

The plasmid pOJ260 is conjugatable into various species of *Streptomyces* and has been shown to incorporate into *Saccharopolyspora spinosa*.<sup>102-104</sup> This is because of the origin of transfer (oriT) derived from the parent plasmid RK2, which allows bacterial conjugation from *Escherichia coli* hosts to a variety of *Streptomyces* species. It also does not contain an origin of replication for *S. spinosa*, which means it cannot be passed on to successive generations of bacteria if it is not incorporated into the genome. So if any
ex-conjugates grow in the presence of hygromycin, they will have incorporated the altered DNA into their genome. Plasmid pOJ260 was used as the parent plasmid in our studies for these reasons.

8.6 Results and Discussion

Unfortunately after the plasmids were made, we were unable to introduce the DNA into wild type *S. spinosa* for the homologous recombination to occur. Several conjugations were performed, yet none were successful. There are several mentions in the literature about methods by which to introduce DNA into *S. spinosa*, but they have yet to be attempted by us.\textsuperscript{102, 104, 105} The two homologous recombination steps and necessary screening are all that remains for the genetic part of this project to be completed. After that, if spinosene is produced by the mutant organism, strain optimization will have to be done and the subsequent chemical modifications could begin. The chemistry behind the library formation of this project is straightforward (Figure 8.8). A variety of *n*-butenyl alcohols will be substituted into the western hemisphere of spinosene through the use of Grubbs-catalyzed metathesis and cross-esterification. By these two chemical reactions, the core structure of the lactone ring would be able to be modified.
Figure 8.8: Chemical Modification of spinosene. Several $n$-butenyl alcohols would be used to replace the western hemisphere of the macrolide ring of spinosene.
CHAPTER 9

CHEMICAL EXPERIMENTALS
9.1 **General Procedures**

All compounds were purchased from Sigma, Acros, Fisher, JT Baker and ASDI, Inc. Solvents were purchased from Fisher. The following solvents were dried based on procedures described in Perrin and Armarego: CH$_2$Cl$_2$ was dried and distilled over CaH$_2$, pyridine was distilled over KOH, benzene was dried over activated 4A molecular sieves, toluene was distilled over phosphorus pentachloride, and THF was dried over sodium metal in the presence of benzophenone. Anhydrous $N,N$-Di-methyl formamide (99.8%) was purchased from Sigma and used without further purification. All other solvents were used without further purification. Sodium azide was re-crystallized from water prior to use. All reactions were performed in flame dried glassware under an atmosphere of argon unless otherwise specified. Ambient temperature is 23 °C. All columns were run on silica gel purchased from Silicycle. Column conditions are reported as a combination of solvents followed by their ratio [e.g. “EtOAc/Hexanes (1:1)” is a 50/50 mixture of ethyl acetate and hexanes]. TLC plates were purchased from Sigma-Aldrich and were UV active. TLCs were stained with a 5 % $p$-anisaldehyde/acid stain in ethanol or 3 % ninhydrin in ethanol.
9.2 Synthesis of Library Compounds

\[
\begin{align*}
\text{OH} & \quad \text{DHP} \\
\text{3} & \quad \text{CH}_2\text{Cl}_2 \\
\text{O} & \quad \text{O} \\
\text{OMe} & \quad \text{OMe}
\end{align*}
\]

4: **Methyl 2-{[5-\text{oxan-2-yl}o\text{xyl}]p\text{ropyl}o\text{xolan}-2-yl}p\text{ropanoate:}** 3,4-Dihydro-2\(H\)-pyran (3.48 g, 41.4 mmol) and \(p\)-toluene sulfonic acid (0.016 g, 0.083 mmol) were added to a stirred solution of 3 (1.79 g, 8.28 mmol) in \(\text{CH}_2\text{Cl}_2\) (33 mL). The solution was stirred at ambient temperature for 7 h. The mixture was then washed with saturated aqueous NaHCO\(_3\) (2 × 20 mL) and water (20 mL). Combined organic fractions were dried over Na\(_2\)SO\(_4\) and concentrated. The crude oil obtained was purified by flash chromatography and eluted in EtOAc/Hexanes (1:9) to give 4 (2.59 g, 99.8%) as a clear oil mixture of diastereomers. \(^1\text{H NMR (400MHz, CDCl}_3\) \(\delta\) 1.11 (d, \(J = 6.4\) Hz, 2H), 1.12 (d, \(J = 6.5\) Hz, 2H), 1.24 (d, \(J = 6.8\) Hz, 2H), 1.46-1.75 (m, 11H), 1.80 (m, 1H), 1.91-2.08 (m, 2H), 2.52 (m, 1H), 3.47 (m, 1H), 3.68 (apparent d, \(J = 4.38\) Hz, 3H), 3.82-4.07 (m, 4H) and 4.68 (dm, 1H); \(^{13}\text{C (100MHz, CDCl}_3\) \(\delta\) 13.32, 13.38, 19.43, 20.22, 22.67, 25.47, 28.58, 31.04, 31.13, 31.34, 31.53, 43.81, 44.09, 45.38, 45.45, 51.57, 63.01, 68.04, 72.62, 76.43, 80.29, 80.39, 94.55, 99.83 and 190.95.
5: 2-{5-[2-(oxan-2-yloxy)propyl]oxolan-2-yl}propan-1-ol: A solution of 4 (2.18 g, 6.93 mmol) in THF (46.0 mL) was cooled to 0 °C on an ice water bath. Lithium aluminum hydride (0.700 g, 18.57 mmol) was slowly added and stirred at ambient temperature for 40 min. Excess LiAlH₄ was neutralized by slowly adding water (0.7 mL) followed by 3.0 M aqueous NaOH (1.4 mL) and more water (0.7 mL). The resulting suspension was mixed with Celite (10 g) and filtered through a glass sintered funnel. The Celite pad was washed with THF (2 × 10 mL) and the solution was concentrated then purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give 5 (1.62 g, 85.6%) as a clear oil. ¹H NMR (400MHz, CDCl₃) δ 0.80 (d, J = 5.9 Hz, 1.5H), 0.81 (d, J = 7.3 Hz, 1.5H), 1.12 (d, J = 6.6 Hz, 1H), 1.25 (d, J = 6.6 Hz, 2H), 1.53 (m, 5H), 1.71 (m, 2H), 1.81 (m, 1H), 1.99 (m, 2H), 3.47 (m, 1H), 3.60 (m, 3H), 3.90 (m, 2H), 4.14 (m, 1H), 4.62 (m, 1H) and 4.68 (m, 1H). IR (film-ATR): 3451, 2938, 2875, 1455, 1374, 1201, 1115, 1075, 1021, 991, 905, 869, 811 and 590 cm⁻¹.
6: 2-{5-[2-(oxan-2-yloxy)propyl]oxolan-2-yl}propyl 4-methylbenzene-1-sulfonate:

5 (0.939 g, 3.45 mmol) was taken up in dry pyridine (4.0 mL). p-Toluene sulfonyl chloride (1.32 g, 6.90 mmol) was added and the mixture was stirred at ambient temperature for 5 h. Water (4 mL) and ether (5 mL) were added and the mixture was washed with saturated aqueous copper sulfate (5 x 5 mL) followed by saturated aqueous NaHCO₃ (5 mL) and water (2 x 5 mL). Combined organic layers were dried over MgSO₄ and concentrated. The residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:6) to give 6 (1.42 g, 96.5%) as a pale yellow oil. ¹H NMR (400MHz, CDCl₃) δ 0.90 (d, J = 6.6, 3H), 1.14 (dd, J = 49.1, 5.9, 3H), 1.56 (m, 11H), 1.74-2.00 (m, 4H), 2.44 (s, 3H), 3.40-3.58 (m, 2H), 3.73-3.99 (m, 4H), 4.16 (ddd, J = 20.5, 9.5, 3.7, 1H), 4.67 (dm, J = 49.1, 1H), 7.33 (d, J = 8.1, 2H) and 7.78 (d, J = 8.1, 2H); ¹³C (100MHz, CDCl₃) δ 13.42, 19.44, 19.54, 20.21, 21.62, 22.67, 25.47, 29.10, 31.04, 31.15, 31.28, 31.42, 38.84, 43.83, 44.13, 48.52, 62.05, 63.01, 67.97, 72.55, 73.14, 73.25, 79.65, 90.18, 94.62, 95.21, 99.78, 106.76, 118.88, 127.91, 129.71, 133.07, 144.48 and 144.54.
7: 2-\{(1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl)oxy\}oxane: Sodium azide (0.705 g, 10.6 mmol) was added to a solution of 6 (2.28 g, 5.34 mmol) in DMF (10.6 mL). The mixture was heated to 95 °C for 1.25 h and cooled to ambient temperature. Ether (10 mL was added and the mixture was washed with water (2 × 10 mL). Combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:9) to give 7 (1.52 g, 96.1%) as a colorless oil. ¹H NMR (400MHz, CDCl₃) δ 0.94 (d, J = 6.6 Hz, 3H), 1.19 (dd, J = 48.4, 5.9 Hz, 3H), 1.40-1.59 (m, 7H), 1.60-1.86 (m, 6H), 1.87-2.07 (m, 2H), 3.22-3.32 (apparent ddd, J = 15.4, 12.5, 7.3 Hz, 1H), 3.40-3.64 (m, 3H), 3.82-3.95 (m, 2H), 4.01 (m, 1H) and 4.72 (dm, J = 38.1 Hz, 1H).

8: 1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-ol: 7 (1.52 g, 5.09 mmol) was taken up in a 0.01 M solution of p-toluene sulfonic acid in MeOH (50.0 mL) and stirred at ambient temperature for 1 h. Saturated NaHCO₃ (3 mL) was added and the mixture was extracted with CHCl₃ (3 × 10 mL). The organic fractions were
combined and washed with brine (3 × 10 mL) and water (10 mL), dried over 
MgSO₄ and concentrated. The residue was purified by flash chromatography and 
eluted with EtOAc/Hexanes (1:3) to give 8 (0.848 g, 79.5%) as a colorless oil. 
¹H 
NMR (400MHz, CDCl₃) δ 0.94 (d, J = 6.6 Hz, 3H), 1.21 (d, J = 6.6 Hz, 3H), 1.50-1.65 
(m, 2H), 1.67 (dd, J = 8.1, 3.7 Hz, 1H), 1.72 (dd, J = 8.1, 4.4 Hz, 1H), 1.78 (m, 1H), 
1.92-2.04 (m, 2H), 2.67 (m, 1H), 3.28 (dd, J = 11.7, 6.6 Hz, 1H), 3.44 (dd, J = 11.7, 
4.4 Hz, 1H), 3.62 (m, 1H) and 3.98-4.17 (m, 2H); ¹³C (100MHz, CDCl₃) δ 14.41, 
23.35, 29.19, 30.64, 39.17, 43.07, 55.09, 65.29, 76.83 and 81.29. IR (film-ATR): 
3387, 2966, 2932, 2095, 1460, 1380, 1282, 1067, 937 and 556 cm⁻¹.

40: 1-{1-[5-{1-azidopropan-2-yl]oxolan-2-yl]propan-2-yl} 4-methyl butanedioate: 8 
(0.106 g, 0.496 mmol) was taken up in pyridine (0.5 mL). Methyl 4-chloro-4-
oxobutyrate (0.116 mL, 0.940 mmol) was added dropwise with rapid stirring. 
The mixture was stirred at ambient temperature for 16 h. Water (3 mL) was 
added and the mixture was extracted with EtOAc (3 × 3 mL). The organic layer 
was washed with saturated aqueous CuSO₄ (4 × 3 mL), NH₄Cl (2 × 3 mL) and brine 
(3 mL), dried over MgSO₄ and concentrated. The residue was purified by flash 
chromatography and eluted in EtOAc/Hexanes (1:6) to give 40 (0.130 g, 79.9%)
as a colorless oil. $^1$H NMR (400MHz, CDCl$_3$) δ 0.93 (d, J = 6.6 Hz, 3H), 1.25 (d, J = 6.6 Hz, 3H), 1.41-1.60 (m, 2H), 1.64-1.82 (m, 3H), 1.87-2.04 (m, 2H), 2.55-2.68 (m, 4H), 3.24 (dd, J = 11.7, 7.3 Hz, 1H), 3.46 (dd, J = 11.7, 4.4 Hz, 1H), 3.55 (q, J = 7.3 Hz, 1H), 3.68 (s, 3H), 3.84 (p, J = 6.6 Hz, 1H) and 5.03 (hex, J = 6.6 Hz, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.42, 20.59, 28.92, 29.16, 29.42, 31.29, 39.45, 42.40, 51.77, 55.08, 69.60, 75.95, 77.00, 80.80, 171.67 and 172.77.

53: 1-methyl 4-[1-(5-{1-[4-(phenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl] butanedioate: A 0.5 M solution of 40 (0.100 mL, 0.050 mmol) in EtOH/H$_2$O (5:1) was mixed with a 0.5 M solution of phenyl-2-propynyl ether 41 (0.200 mL, 0.100 mmol) in EtOH/H$_2$O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0045 mL, 0.005 mmol) and a 0.3 M aqueous solution of copper sulfate pentahydrate [CuSO$_4$•5H$_2$O] (0.0017 mL, 0.500 µmol) were added and the mixture was stirred at ambient temperature for 42 h. The reaction was still incomplete as determined by TLC (EtOAc/Hexanes 1:1, p-anisaldehyde stain). More CuSO$_4$•5H$_2$O (0.0135 mL, 4.10 µmol) and ascorbate (0.0045 mL, 0.005 mmol) were added and the mixture was stirred for a further 3 d. Upon completion as determined by TLC, saturated NaHCO$_3$ (1 mL) was added
and the mixture was extracted with EtOAc (3 × 1 mL). Combined organic layers were concentrated, resuspended in CHCl₃ and concentrated again to give a crude residue which was purified by chromatography and eluted in EtOAc/Hexanes (1:1) to give 53 (0.015 g, 65.2%). ¹H NMR (400MHz, CDCl₃) δ 0.85 (d, J = 7.3 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.68 (ddd, J = 13.9, 8.1, 4.4 Hz, 1H), 1.80 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.90-2.05 (m, 3H), 2.56-2.67 (m, 4H), 3.33-3.41 (m, 1H), 3.66 (s, 3H), 3.78-3.87 (m, 1H), 4.32 (dd, J = 13.9, 7.3 Hz, 1H), 4.55 (dd, J = 13.9, 4.4 Hz, 1H), 5.08-5.17 (m, 1H), 5.21 (s, 2H), 6.92-7.02 (m, 3H), 7.25-7.31 (m, 2H) and 7.69 (s, 1H); ¹³C (100MHz, CDCl₃) δ 14.45 (CH₃), 20.73 (CH₃), 28.89 (CH₂), 29.38 (CH₂), 29.55 (CH₂), 31.23 (CH₂), 40.28 (CH), 42.37 (CH₂), 51.77 (CH₃), 53.37 (CH₂), 61.97 (CH₂), 69.28 (CH), 75.98 (CH), 80.63 (CH), 114.82 (CH), 121.08 (CH), 123.91 (CH), 129.43 (CH), 143.82 (quat), 158.27 (quat), 171.72 (C=O) and 172.74 (C=O). IR (film-ATR): 2951, 2877, 1729, 1599, 1587, 1495, 1217, 1162, 1049, 755 and 693 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₉H₃₄N₃O₆: 460.5523; found 460.5515 ([M + H]⁺, Δppm 1.8).

\[
\begin{align*}
\text{MeO} & \quad \text{O} \\
\begin{array}{c}
\text{O} \\
\text{N}_3
\end{array} & \quad \begin{array}{c}
\text{O} \\
\text{N}_3
\end{array} \\
\text{40} & \quad \begin{array}{c}
\text{O} \\
\text{N}_3
\end{array} \\
\text{CuSO}_4 \cdot 5\text{H}_2\text{O} & \quad \text{ascorbate} \\
\text{EtOH-H}_2\text{O 5:1}
\end{align*}
\]

54: 1-methyl 4-[1-(5-{1-[4-(4-methylphenoxymethyl)-1H,1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl] butanedioate: A 0.5 M solution of 40 (0.100 mL, 121
0.05 mmol) in EtOH/H_2O (5:1) was mixed with a 0.5 M solution of \textit{p}-tolyl-2-propargyl ether 42 (0.200 mL, 0.10 mmol) in EtOH/H_2O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.009 mL, 0.010 mmol) and a 0.3 M aqueous solution of CuSO_4\cdot5H_2O (0.0152 mL, 0.005 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO_3 (2 mL) was added and the suspension was extracted with EtOAc (4 × 2 mL). The combined organic layers were washed with brine (2 mL) and water (2 mL) then dried over Na_2SO_4. The cloudy mixture was concentrated and the residue was purified by column chromatography and eluted in EtOAc/Hexanes (2:3) to give 54 (0.017 g, 71.7%). ^1H NMR (400MHz, CDCl_3) δ 0.84 (d, J = 6.6 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.45-1.60 (m, 2H), 1.68 (apparent dq, J = 13.9, 4.4 Hz, 1H), 1.80 (apparent dq, J = 13.9, 4.4 Hz, 1H), 2.28 (s, 3H), 1.91-2.05 (m, 3H), 2.61 (br m, 4H), 3.34-3.42 (m, 1H), 3.66 (s, 3H), 3.78-3.87 (m, 1H), 4.31 (dd, J = 13.9, 7.3 Hz, 1H), 4.55 (dd, J = 13.9, 4.4 Hz, 1H), 5.07-5.16 (m, 1H), 5.18 (s, 2H), 6.88 (d, J = 8.8 Hz, 2H), 7.07 (d, J = 8.8 Hz, 2H) and 7.67 (s, 1H); ^13C (100MHz, CDCl_3) δ 14.42, 20.46, 20.70, 28.89, 29.41, 29.55, 31.24, 40.28, 42.37, 51.80, 53.36, 62.17, 69.31, 75.98, 80.64, 114.68, 123.86, 129.87, 130.32, 143.99, 156.17, 171.72, and 172.77. IR (film-ATR): 2952, 1731, 1438, 1364, 1217, 1162, 1048 and 815 cm\textsuperscript{-1}. HRMS (ESI) \textit{m/z} calcd. for C_{23}H_{36}N_3O_6: 474.5793; found 474.5787 ([M + H]^+, Δ ppm 1.3).
General Procedure A: Huisgen 1,3-dipolar cycloaddition reactions for SAR Round 1 propargyl ethers

\[
\text{R}_1\text{O} \rightleftharpoons \text{O} \rightleftharpoons \text{O} \rightleftharpoons \text{N}_3 + \equiv \text{R}_2 \xrightleftharpoons{\text{CuSO}_4, \text{ascorbate, EtOH/H}_2\text{O} (5:1)} \text{R}_1\text{O} \rightleftharpoons \text{O} \rightleftharpoons \text{O} \rightleftharpoons \text{N} \equiv \text{N} \rightleftharpoons \text{R}_2
\]

55-68: A 0.5 M solution of azide (0.100 mL, 0.05 mmol) in EtOH/H$_2$O (5:1) was mixed with a 0.5 M solution of alkyne (0.200 mL, 0.10 mmol) in EtOH/H$_2$O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.009 mL, 0.010 mmol) and a 0.3 M aqueous solution of CuSO$_4$$\cdot$5H$_2$O (0.0152 mL, 0.005 mmol) were added. The mixture generally changed color upon the addition of the copper sulfate and often a precipitate formed. This mixture was stirred at ambient temperature for 18 h. Upon completion as determined by TLC (EtOAc/Hexanes 5:3, $p$-anisaldehyde stain), EtOAc (2 mL) and saturated aqueous NaHCO$_3$ (2 mL) were added and the mixture was extracted with EtOAc (3 x 2 mL). Combined organic layers were washed with brine (2 mL) and water (2 mL) then dried over MgSO$_4$ and concentrated. Each compound was purified by chromatography and eluted in varying ratios of EtOAc/Hexanes.
55: 1-[1-(5-{1-[4-(4-ethylphenoxy)methyl]-1H-1,2,3-triazol-1-yl}propan-2-yl)propan-2-yl] 4-methyl butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-ethynyl-4-(prop-2-yn-1-yloxy)benzene 43 as the alkyne. The residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 55 (0.0197 g, 80.7%).

$^1$H NMR (400MHz, CDCl$_3$) δ 0.84 (d, J = 7.3 Hz, 3H), 1.20 (t, J = 7.3 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.45-1.60 (m, 2H), 1.69 (apparent dq, J = 13.9 Hz, 4.4 Hz, 1H), 1.80 (apparent dq, J = 13.9, 4.4 Hz, 1H), 1.91-2.05 (m, 3H), 2.58 (q, J = 7.3 Hz, 2H), 2.60 (s, 4H), 3.35-3.42 (m, 1H), 3.66 (s, 3H), 3.78-3.88 (m, 1H), 4.31 (dd, J = 13.9, 7.3 Hz, 1H), 4.55 (dd, J = 13.9, 4.4 Hz, 1H), 5.07-5.16 (m, 1H), 5.19 (s, 2H), 6.91 (d, J = 8.8 Hz, 2H), 7.10 (d, J = 8.8 Hz, 2H) and 7.68 (s, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.42, 15.83, 20.70, 27.96, 28.89, 29.42, 29.56, 31.24, 40.28, 42.37, 51.80, 53.37, 62.17, 69.31, 75.98, 80.64, 114.71, 123.84, 128.69, 136.84, 144.01, 156.34, 171.73 and 172.74. IR (film-ATR): 2964, 1731, 1511, 1438, 1364, 1217, 1162, 1048 and 829 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{26}$H$_{36}$N$_3$O$_6$: 488.6063; found 488.6048 ([M + H]$^+$, Δppm 3.0).
56: 1-[1-(5-{1-[4-(4-methoxyphenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl] 4-methyl butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-methoxy-4-(prop-2-yn-1-yloxy)benzene 44 as the alkyne. The crude solid was purified by column chromatography and eluted in EtOAc/Hexanes (2:1) to give 56 (0.0186 g, 75.9%). \(^1\)H NMR (400MHz, CDCl\(_3\)) \(\delta\) 0.84 (d, J = 6.6 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.45-1.60 (m, 2H), 1.68 (ddd, J = 13.9, 8.8, 4.4 Hz 1H), 1.80 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.92-2.02 (m, 3H), 2.61 (m, 4H), 3.37 (m, 1H), 3.66 (s, 3H), 3.76 (s, 3H), 3.82 (m, 1H), 4.31 (dd, J = 13.2, 7.3 Hz, 1H), 4.54 (dd, J = 13.9, 4.4 Hz, 1H), 5.12 (m, 1H), 5.15 (s, 2H), 6.82 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H) and 7.67 (s, 1H); \(^{13}\)C (100MHz, CDCl\(_3\)) \(\delta\) 14.42, 20.73, 28.89, 29.38, 29.56, 31.24, 40.28, 42.37, 51.80, 53.35, 55.66, 62.75, 69.28, 75.98, 80.61, 114.57, 115.93, 123.89, 143.99, 152.40, 154.05, 171.75 and 172.77. IR (film-ATR): 2954, 1729, 1507, 1462, 1365, 1217, 1162, 1036 and 826 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for C\(_{25}\)H\(_{36}\)N\(_3\)O\(_7\): 490.5786; found 490.5773 ([M + H]\(^+\), \(\Delta\)ppm 2.7).
57: 1-[1-{5-{1-[4-(3-methoxyphenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl]propan-2-yl] 4-methyl butanedioate: This reaction was performed as described in General Procedure A with the exception that the reaction was stirred for 6 d. Compound 40 was used as the azide and 1-methoxy-3-(prop-2-yn-1-ylxy)benzene 45 was used as the alkyne. The crude solid was purified by column chromatography and eluted in EtOAc/Hexanes (5:3) to give 57 (0.0116 g, 47.3%). $^1$H NMR (400MHz, CDCl$_3$) $\delta$ 0.85 (d, J = 6.6 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.68 (ddd, J = 13.2, 8.1, 4.4 Hz, 1H), 1.80 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.90-2.05 (m, 3H), 2.60 (s, 3H), 3.38 (m, 1H), 3.66 (s, 3H), 3.78 (s, 3H), 3.82 (m, 1H), 4.32 (dd, J = 13.2, 7.3 Hz, 1H), 4.55 (dd, J = 13.9, 3.7 Hz, 1H), 5.12 (m, 1H), 5.19 (s, 2H), 6.50-6.62 (m, 3H), 7.18 (t, J = 8.1 Hz, 1H) and 7.70 (s, 1H); $^{13}$C (100MHz, CDCl$_3$) $\delta$ 14.43, 20.71, 28.88, 29.39, 29.55, 31.22, 40.25, 42.35, 51.78, 53.36, 55.23, 62.02, 69.25, 75.96, 77.19, 80.60, 101.27, 106.82, 123.96, 129.85, 143.67, 159.52, 160.75, 171.74 and 172.75. IR (NaCl): 2964, 1734, 1593, 1493, 1457, 1264, 1199, 1153, 1049 and 688 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{25}$H$_{36}$N$_3$O$_7$: 490.5786; found 490.5772 ([M + H]$^+$, $\Delta$ppm 2.9).
1-methyl 4-[1-{5-[1-{4-(4-nitrophenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl]propan-2-yl] butanedioate: This reaction was performed as described in General Procedure A with the exceptions that solubility issues with the alkyne, 1-nitro-4-(prop-2-yn-1-yloxy)benzene 46 required using t-BuOH/H₂O (4:1) instead of EtOH/H₂O (5:1) to solvate the alkyne and the mixture was stirred for 5 d before being worked up according to General Procedure A. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (5:3) to give 58 (0.013 g, 51.6%). ¹H NMR (400MHz, CDCl₃) δ 0.87 (d, J = 6.6 Hz, 3H), 1.25 (d, J = 6.6 Hz, 3H), 1.46-1.59 (m, 2H), 1.66 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.81 (ddd, J = 14.7, 8.8, 3.7 Hz, 1H), 1.91-2.03 (m, 3H), 2.61 (m, 4H), 3.32 (m, 1H), 3.65 (s, 3H), 3.81 (m, 1H), 4.39 (dd, J = 13.2, 6.6 Hz, 1H), 4.53 (dd, J = 13.9, 3.7 Hz, 1H), 5.16 (m, 1H), 5.31 (s, 2H), 7.10 (dm, J = 8.8 Hz, 2H), 7.80 (s, 1H) and 8.20 (dm, J = 8.8 Hz, 2H); ¹³C (100MHz, CDCl₃) δ 14.58, 20.79, 28.89, 29.38, 29.72, 31.18, 40.28, 42.39, 51.80, 53.45, 62.36, 69.11, 75.87, 77.19, 80.58, 114.91, 124.72, 125.88, 141.73, 142.20, 163.29 and 171.81. IR (film-ATR): 2973, 1729, 1592, 1513, 1340, 1255, 1163, 1111, 991, 847 and 753 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₄H₃₃N₄O₆: 505.5497; found 505.5500 ([M + H]⁺, Δppm 0.5).
59: 1-[1-(5-{1-[4-(cyanophenoxy)methyl]-1H-1,2,3-triazol-1-yl}propan-2-yl)oxolan-2-yl]propan-2-yl] 4-methyl butanedioate: A 0.5 M solution of 40 (0.100 mL, 0.050 mmol), a 1.1 M aqueous solution of ascorbate (0.009 mL, 0.01 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.015 mL, 0.005 mmol) were added to a 0.5 M solution of 4-(prop-2-yn-1-yl)oxazoline 47 (0.500 mL, 0.250 mmol) in t-BuOH/H₂O (4:1) and the mixture was stirred at ambient temperature for 72 h. The reaction was incomplete as determined by TLC (EtOAc/Hexanes 5:3, p-anisaldehyde stain). The usual workup yielded 0.005 g of product. The azide 40 and alkyne 47 were recovered from the column and were taken back up in EtOH/H₂O (0.750 mL). A 1.1 M aqueous solution of ascorbate (0.009 mL, 0.010 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.015 mL, 0.005 mmol) were again added and the mixture was stirred at ambient temperature for 18 h. EtOAc (2 mL) and saturated aqueous NaHCO₃ (2 mL) were added and the mixture was extracted with EtOAc (3 × 2 mL). Combined organic layers were washed with brine (2 mL) and water (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (5:4) to give 59, when combined with the yield from the first attempt, gave (0.017 g, 70.2%). ¹H NMR (400MHz, CDCl₃) δ 0.86 (d, J = 6.6 Hz,
3H), 1.25 (d, J = 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.66 (ddd, J = 13.9, 8.8, 3.7 Hz, 1H), 1.81 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.92-2.03 (m, 3H), 2.60 (m, 4H), 3.33 (m, 1H), 3.65 (s, 3H), 3.81 (m, 1H), 4.37 (dd, J = 13.9, 6.6 Hz, 1H), 4.53 (dd, J = 13.2, 3.7 Hz, 1H), 5.15 (m, 1H), 5.26 (s, 2H), 7.08 (dm, J = 8.8 Hz, 2H), 7.58 (dm, J = 8.8 Hz, 2H) and 7.77 (s, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.56, 20.79, 28.89, 29.38, 29.69, 31.18, 40.28, 42.39, 51.80, 53.45, 62.03, 69.14, 75.89, 80.58, 104.32, 115.59, 119.12, 124.58, 133.95, 142.42, 161.55, 171.78 and 172.72. IR (film-ATR): 2933, 2224, 1728, 1604, 1575, 1508, 1254, 1171, 1050, 995 and 837 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{25}$H$_{33}$N$_4$O$_6$: 485.5621; found 485.5623 ([M + H]$^+$, Δppm 0.3).

![Chemical Structure Image]

60: 1-[1-(5-[1-[4-(3-fluorophenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl)propan-2-yl] 4-methyl butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-fluoro-3-(prop-2-yn-1-yloxy)benzene 48 as the alkyne. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (5:4) to give 60 (0.0138 g, 57.7%). $^1$H NMR (400MHz, CDCl$_3$) δ 0.85 (d, J = 6.6 Hz, 3H), 1.25 (d, J = 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.67 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.80 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.91-2.03 (m, 3H), 2.60 (m, 4H), 3.35 (m, 1H), 3.66 (s, 3H), 3.81 (m, 1H), 4.34 (dd, J = 13.9, 6.6 Hz, 1H), 4.54 (dd, J = 13.9, 3.7 Hz, 1H), 5.13
(m, 1H), 5.19 (s, 2H), 6.62-6.81 (m, 3H), 7.23 (m, 1H) and 7.73 (s, 1H); $^{13}$C
(100MHz, CDCl$_3$) δ 14.47, 20.73, 28.89, 29.39, 29.63, 31.21, 40.27, 42.38, 51.80,
53.37, 62.17, 69.22, 75.93, 80.58, 102.58, 102.83, 107.79, 108.02, 110.48,
124.22, 130.15, 130.26, 143.15, 159.57, 159.68, 162.30, 164.72, 171.78, 172.77
and 195.57. HRMS (ESI) $m/z$ calcd. for C$_{24}$H$_{33}$N$_3$O$_6$: 478.5427; found 478.5411
([M + H]$^+$, Δppm 3.4).

![Chemical Reaction Diagram]

61: 1-[1-(5-{1-[4-(4-chlorophenoxymethyl)-1H-1,2,3-triazol-1-yl]propan-2-
yl}oxolan-2-yl)propan-2-yl] 4-methyl butanedioate: This reaction was
performed as described in General Procedure A using 40 as the azide and 1-
chloro-4-(prop-2-yn-1-yloxy)benzene 49 as the alkyne. The crude residue was
purified by column chromatography and eluted in EtOAc/Hexanes (5:4) to give
61 (0.018 g, 72.9%). $^1$H NMR (400MHz, CDCl$_3$) δ 0.84 (d, J = 6.6 Hz, 3H), 1.25 (d, J
= 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.67 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.80 (ddd, J
= 13.9, 8.8, 4.4 Hz, 1H), 1.91-2.04 (m, 3H), 2.60 (m, 4H), 3.34 (m, 1H), 3.66 (s, 3H),
3.82 (m, 1H), 4.34 (dd, J = 13.9, 7.3 Hz, 1H), 4.53 (dd, J = 13.9, 4.4 Hz, 1H), 5.13
(m, 1H), 5.18 (s, 2H), 6.93 (dm, J = 8.8 Hz, 2H), 7.22 (dm, J = 8.8 Hz, 2H) and 7.71
(s, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.47, 20.76, 28.89, 29.39, 29.61, 31.21, 40.25,
42.37, 51.80, 53.37, 62.19, 69.22, 75.92, 80.58, 116.20, 124.19, 125.95, 129.30, 143.27, 156.86, 171.75 and 172.74. IR (film-ATR): 2952, 1729, 1596, 1538, 1491, 1461, 1438, 1364, 1281, 1239, 1163, 1092, 1049, 1002 and 825 cm⁻¹. ESI⁺MS m/z 495 [M+H]⁺, 497 [M+2]⁺.

62: 1-methyl 4-[1-{5-[1-[4-(phenylphenoxy)methyl]-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl]propan-2-yl] butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-phenyl-4-(prop-2-yn-1-yloxy)benzene 50 as the alkyne with the exception that the mixture was stirred for 3 d. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (5:4) to give 62 (0.0186 g, 69.4%). ¹H NMR (400MHz, CDCl₃) δ 0.86 (d, J = 6.6 Hz, 3H), 1.26 (d, J = 6.6 Hz, 3H), 1.45-1.60 (m, 2H), 1.68 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.81 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.91-2.05 (m, 3H), 2.61 (m, 4H), 3.37 (m, 1H), 3.66 (s, 3H), 3.83 (m, 1H), 4.34 (dd, J = 13.2, 7.3 Hz, 1H), 4.56 (dd, J = 13.9, 3.7 Hz, 1H), 5.13 (m, 1H), 5.26 (s, 2H), 7.07 (dm, J = 8.8 Hz, 2H), 7.27-7.33 (m, 1H), 7.38-7.44 (m, 2H), 7.49-7.57 (m, 4H) and 7.73 (s, 1H); ¹³C (100MHz, CDCl₃) δ 14.47, 20.73, 28.89, 29.38, 29.58, 31.23, 40.28, 42.37, 51.80, 53.40, 62.11, 69.28, 75.94, 80.63, 100.46, 115.24, 124.05, 126.70, 128.11,
128.69, 134.12, 140.64, 143.74, 157.86, 171.78 and 172.77. IR (film-ATR): 2958, 1731, 1609, 1585, 1522, 1491, 1453, 1367, 1317, 1271, 1246, 1164, 1056, 1009, 838, 766 and 697 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for \(\text{C}_{30}\text{H}_{38}\text{N}_{3}\text{O}_{6}\): 536.6504; found 536.6544 ([\(\text{M} + \text{H}\)]\(^+\), \(\Delta\text{ppm}\) 7.4).

![Chemical structure](image)

63: \(\text{1-[1-[(5-[^{4}\text{bromophenoxy}]methyl)-1\text{H}-1,2,3-\text{triazol}-1-\text{yl}]propan-2-yl} \text{oxolan-2-yl} \text{propan-2-yl}]\) 4-methyl butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-bromo-4-(prop-2-yn-1-yl)oxy]benzene 51 as the alkyne with the exception that the mixture was stirred for 2 d. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (5:4) to give 63 (0.0175 g, 65.1%).

\(^{1}\text{H NMR (400MHz, CDCl}_{3}\)} \(\delta\) 0.85 (d, \(J = 6.6\) Hz, 3H), 1.25 (d, \(J = 6.6\) Hz, 3H), 1.44-1.60 (m, 2H), 1.67 (ddd, \(J = 13.9, 8.8, 4.4\) Hz, 1H), 1.80 (ddd, \(J = 13.9, 8.8, 4.4\) Hz, 1H), 1.92-2.04 (m, 3H), 2.60 (m, 4H), 3.34 (m, 1H), 3.66 (s, 3H), 3.81 (m, 1H), 4.34 (dd, \(J = 13.9, 7.3\) Hz, 1H), 4.53 (dd, \(J = 13.9, 4.4\) Hz, 1H), 5.13 (m, 1H), 5.18 (s, 2H), 6.89 (dm, \(J = 8.8\) Hz, 2H), 7.37 (dm, \(J = 8.8\) Hz, 2H) and 7.70 (s, 1H); \(^{13}\text{C (100MHz, CDCl}_{3}\)} \(\delta\) 14.48, 20.76, 28.89, 29.39, 29.61, 31.21, 40.25, 42.37, 51.80, 53.38, 62.14, 69.23, 75.93, 80.58, 113.28, 116.73, 124.22, 132.25, 143.25, 157.36, 171.78 and 172.77. IR (film-ATR): 2953, 1729, 1590, 1488, 1461, 1437, 1363, 132
1283, 1239, 1162, 1072, 1049, 999, 910, 822 and 596 cm$^{-1}$. ESI$^+$MS $m/z$ 538 (M + H)$^+$, 540 (M + 2)$^+$: HRMS (ESI) $m/z$ calcd. for C$_{24}$H$_{32}$N$_3$O$_6$Br: 538.4399; found 538.5148 ([M]$^+$, Δppm 139.1).

64: $1$-[5-$[1$-$4$-iodophenoxymethyl]-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl] 4-methyl butanedioate: This reaction was performed as described in General Procedure A using 40 as the azide and 1-ido-4-(prop-2-yn-1-yloxy)benzene 52 as the alkyne with the exception that the mixture was stirred for 2 d. The crude residue was purified by chromatography and eluted in EtOAc/Hexanes (5:4) to give 64 (0.0247 g, 84.3%). $^1$H NMR (400MHz, CDCl$_3$) δ 0.85 (d, J = 6.6 Hz, 3H), 1.25 (d, J = 6.6 Hz, 3H), 1.44-1.60 (m, 2H), 1.67 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.80 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.92-2.05 (m, 3H), 2.60 (m, 4H), 3.34 (m, 1H), 3.66 (s, 3H), 3.81 (m, 1H), 4.34 (dd, J = 13.9, 6.6 Hz, 1H), 4.53 (dd, J = 13.2, 4.4 Hz, 1H), 5.13 (m, 1H), 5.17 (s, 2H), 6.78 (dm, J = 8.8 Hz, 2H), 7.55 (dm, J = 8.8 Hz, 2H) and 7.70 (s, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.47, 20.76, 28.89, 29.39, 29.61, 31.21, 40.25, 42.37, 51.80, 53.37, 62.00, 69.22, 75.92, 80.58, 83.25, 117.30, 124.18, 138.20, 143.21, 158.16, 171.78 and 172.74. IR (film-ATR): 2952, 2876, 1728, 1584, 1573, 1485, 1462, 1439, 1365, 1280, 1239, 1162, 1049, 997, 820, 754 and 583 cm$^{-1}$.
65: **1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl benzoate:** Benzoyl chloride (0.088 mL, 0.760 mmol) was added dropwise to a solution of 8 (0.080 g, 0.375 mmol) in pyridine (0.40 mL) with vigorous stirring. The mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (3 mL) and EtOAc (3 mL) were added and the mixture was washed with saturated aqueous NH₄Cl (2 × 3 mL) and water (2 × 3 mL). Combined organic fractions were dried over MgSO₄. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:6) to give 65 (0.0653 g, 54.1%).

66: **1-(5-{1-[4-(4-phenylphenoxy)methyl]-1H-1,2,3-triazol-1-yl}propan-2-yl)oxolan-2-yl)propan-2-yl benzoate:** This reaction was performed as described in General Procedure A using 65 as the azide and 1-phenyl-4-{prop-2-yn-1-yloxy}benzene 50 as the alkyne. The crude residue was purified by chromatography and eluted in EtOAc/Hexanes (1:2) to give 66 (0.183 g, 69.6%). IR (film-ATR): 3142, 3061, 3031, 2970, 2875, 1711, 1608, 1584, 1518, 1486, 1451, 1385, 1356, 1314, 1271, 1243,
1176, 1110, 1069, 1026, 1003, 911, 833, 763, 713 and 698 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for \(C_{32}H_{36}N_3O_4\): 526.2706; found 526.2722 ([M + H]\(^+\), \(\Delta\)ppm 3.1).

\[
\text{C}_{32}\text{H}_{36}\text{N}_3\text{O}_4
\]

67: \(1-(5\{}\{1\{}[4\{}-(4\text{-ethylphenoxy)methyl\}]-1H,1,2,3\text{-triazol}-1\text{-yl}\}propan-2\text{-yl}\}\)oxolan-2-yl)propan-2-yl benzoate: This reaction was performed as described in General Procedure A using 65 as the azide and 1-ethyl-4-(prop-2-yn-1-yloxy)benzene 43 as the alkyne. The crude residue was purified by chromatography and eluted in EtOAc/Hexanes (1:2) to give 67 (0.015 g, 62.8%). IR (film-ATR): 2965, 2875, 1712, 1611, 1584, 1511, 1461, 1451, 1384, 1314, 1276, 1177, 1110, 1069, 1048, 1026, 829, 713 and 688 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for \(C_{28}H_{36}N_3O_4\): 478.2706; found 478.2697 ([M + H]\(^+\), \(\Delta\)ppm 1.8).

\[
\text{C}_{28}\text{H}_{36}\text{N}_3\text{O}_4
\]

68: \(1-(5\{}\{1\{}[4\{}-(3\text{-fluorophenoxy)methyl\}]-1H,1,2,3\text{-triazol}-1\text{-yl}\}propan-2\text{-yl}\}\)oxolan-2-yl)propan-2-yl benzoate: This reaction was performed as described in General
Procedure A using 65 as the azide and 1-fluoride-3-(prop-2-yn-1-yloxy)benzene 48 as the alkyne. The crude residue was purified by chromatography and eluted in EtOAc/Hexanes (1:2) to give 68 (0.0184 g, 78.6%). IR (film-ATR): 3142, 3072, 2967, 2877, 1712, 1613, 1592, 1489, 1451, 1386, 1314, 1276, 1166, 1133, 1110, 1070, 1049, 1026, 995, 959, 931, 846, 769, 712 and 681 cm⁻¹. HRMS (ESI) m/z calcd. for C_{26}H_{31}N_{3}O_{4}F: 468.2299; found 468.2331 ([M + H]^+, Δppm 6.9).

![Chemical structure](image)

69: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-ol: A solution of 2-ethynyl pyridine (0.231 g, 2.24 mmol) in EtOH/H_2O (5:1, 6.0 mL) was added to a stirred solution of 8 (0.318 g, 1.49 mmol) in EtOH/H_2O (5:1, 3.0 mL). A 1.1 M aqueous solution of ascorbate (0.455 mL, 0.500 mmol) and a 0.3 M aqueous solution of CuSO_4•5H_2O (5.5 mL, 1.64 mmol) were added and the mixture was stirred at ambient temperature until completed as determined by TLC (EtOAc/Hexanes 1:1, p-anisaldehyde stain). EtOAc (5 mL) and saturated aqueous NaHCO_3 (5.0 mL) were added and the mixture was extracted with EtOAc (3 × 5 mL). Combined organic layers were washed with brine (5 mL), dried over MgSO_4 and concentrated. The crude residue was purified by flash.
chromatography and eluted in EtOAc/Hexanes (7:1) to give 69 (0.342 g, 72.6%) as a white crystalline solid.

88: 1-(5-1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl)oxolan-2-yl 3-chloro-6-fluoro-1-benzo thiophene-2-carboxylate: A 1.0 M solution of 3-chloro-6-fluoro-1-benzo thiophene-2-carbonyl chloride 70 (0.0149 g, 0.060 mmol) in CH$_2$Cl$_2$ (0.060 mL) was added to 69 (0.0082 g, 0.026 mmol). Pyridine (0.50 mL) was added and the mixture was heated on a J-KEM reaction block (176 °C bottom, 10 °C top) for 30 min. The mixture was taken up in EtOAc (3 mL) and washed with saturated aqueous copper sulfate (5 × 2 mL) and brine (3 mL), dried over MgSO$_4$ and concentrated. The pale green solid was purified by column chromatography and eluted in EtOAc/Hexanes (3:5) to give 88 (0.0062 g, 44.9%).

$^1$H NMR (400MHz, CDCl$_3$) δ 0.86 (d, J = 6.6 Hz, 3H), 1.44 (d, J = 6.6 Hz, 3H), 1.60 (m, 2H), 1.88 (ddd, J = 13.9, 8.1, 4.4 Hz, 1H), 1.95-2.13 (m, 4H), 3.54 (m, 1H), 4.03 (m, 1H), 4.33 (dd, J = 13.9, 8.1 Hz, 1H), 4.66 (dd, J = 13.9, 4.4 Hz, 1H), 5.37 (m, 1H), 7.20 (m, 1H), 7.22 (dd, J = 8.8, 2.2 Hz, 1H), 7.49 (dd, J = 8.8, 2.2 Hz, 1H), 7.76 (m, 1H), 7.92 (dd, J = 8.8, 5.1 Hz, 1H), 8.14 (br s, 1H), 8.16 (s, 1H) and 8.53 (m, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.28, 20.79, 29.55, 31.40, 40.43, 42.45, 53.53, 71.07, 76.09, 77.19, 80.83, 108.62, 108.87, 114.90, 115.15, 120.22, 122.65,
122.79, 125.44, 125.52, 126.84, 127.45, 136.79, 149.34, 160.50 and 163.95. IR (film-ATR): 3071, 2963, 2930, 2876, 1778, 1718, 1605, 1566, 1517, 1467, 1421, 1356, 1332, 1308, 1284, 1241, 1200, 1145, 1078, 1040, 996, 977, 946, 894, 873, 847, 811, 785 and 760 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₆H₂₇N₂O₃FSCl: 529.1476; found 529.1479 ([M + H]⁺, Δppm 0.5).

89: 1-[(5-1-[4-(pyridin-2-yl)]-1H-1,2,3-triazol-1-yl)propan-2-yl]oxolan-2-yl)propan-2-yl 2-chloro-4-nitrobenzoate: A solution of 2-chloro-4-nitrobenzoyl chloride 71 (0.011 g, 0.050 mmol) in CH₂Cl₂ (0.025 mL) was added to a solution of 69 (0.0095 g, 0.030 mmol) in pyridine (0.030 mL). More pyridine (0.500 mL, 6.18 mmol) was added and the mixture was heated to 50 °C for 24 h. CH₂Cl₂ (3 mL) was added and the mixture was washed with saturated aqueous CuSO₄ (4 × 2 mL), saturated aqueous NH₄Cl (2 mL), water (2 mL) and brine (2 mL), dried over MgSO₄ and concentrated. The crude, pale green solid was purified by chromatography and eluted in EtOAc/Hexanes (3:5) to give 89 (0.0051 g, 34.0%).

¹H NMR (400MHz, CDCl₃)  δ 0.88 (d, J = 6.6 Hz, 3H), 1.45 (d, J = 6.6 Hz, 3H), 1.52-1.70 (m, 3H), 1.84 (ddd, J = 13.9, 8.8, 4.4 Hz, 1H), 1.95-2.16 (m, 4H), 3.55 (m, 1H), 4.00 (m, 1H), 4.36 (dd, J = 13.9, 8.1 Hz, 1H), 4.66 (dd, J = 13.9, 4.4 Hz, 1H), 5.43
(m, 1H), 7.22 (m, 1H), 7.77 (m, 1H), 7.95 (d, J = 8.8 Hz, 1H), 8.13-8.22 (m, 3H), 8.31 (d, J = 2.2 Hz, 1H) and 8.56 (br s, 1H); $^{13}$C (100MHz, CDCl$_3$) δ 14.31, 20.65, 29.47, 29.69, 31.35, 40.39, 42.40, 53.53, 71.87, 75.81, 77.19, 80.91, 120.27, 120.33, 121.46, 122.73, 122.84, 125.93, 131.80, 136.63, 136.84, 148.11, 149.31 and 163.87. IR (film-ATR): 3104, 2962, 2926, 2855, 1730, 1603, 1571, 1526, 1464, 1421, 1384, 1349, 1286, 1246, 1204, 1141, 1109, 1041, 996, 977, 894, 842, 785, 758 and 734 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{24}$H$_{27}$N$_3$O$_5$Cl: 500.1701; found 500.1693 ([M + H]$^+$, Δppm 1.5).

90: **1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 4-benzylmorpholine-2-carboxylate:** A 1.0 M solution of 69 (0.0096 g, 0.030 mmol) in pyridine (0.030 mL) was added to N-benzyl morpholino-2-carbonyl chloride 72 (0.0120 g, 0.050 mmol). More pyridine (0.500 mL, 6.18 mmol) was added and the mixture was heated on a J-KEM reaction block (125 °C bottom, 10 °C top) for 72 h. The reaction was cooled to ambient temperature and saturated aqueous NaHCO$_3$ (2 mL) was added. The mixture was then extracted with ether (3 × 2 mL). Combined organic fractions were washed with water (3 × 2 mL) dried over MgSO$_4$ and concentrated. The crude product was purified by chromatography and eluted in EtOAc containing 5% v/v Et$_3$N to give 90 in quantitative yield. $^1$H NMR (400MHz, CDCl$_3$) δ 0.86 (apparent dd, J = 7.0, 1.8 Hz,
3H), 1.28 (apparent t, J = 6.1 Hz, 3H), 1.42-1.63 (m, 2H), 1.71 (m, 1H), 1.83 (m, 1H), 1.90-2.00 (m, 2H), 2.05 (m, 1H), 2.25-2.45 (m, 2H), 2.56 (m, 1H), 2.93 (m, 1H), 3.41-3.61 (m, 4H), 3.68 (m, 1H), 3.86 (m, 1H), 4.01 (m, 1H), 4.23 (m, 1H), 4.33 (m, 1H), 4.64 (apparent dt, J = 14.0, 4.4 Hz, 1H), 5.18 (m, 1H), 7.18-7.29 (m, 3H), 7.29-7.32 (m, 3H), 7.76 (m, 1H), 8.16 (m, 1H) and 8.58 (m, 1H); 13C

(100MHz, CDCl3) δ 14.17, 14.31, 20.68, 29.50, 31.26, 40.44, 42.32, 42.37, 52.38, 53.55, 54.91, 62.99, 66.08, 66.24, 69.99, 70.08, 74.43, 74.62, 75.98, 76.03, 76.86, 77.22, 80.75, 80.80, 120.23, 122.68, 122.90, 127.26, 128.28, 129.01, 136.82, 137.37, 148.12, 149.37, 150.44, 169.68 and 187.44. IR (film-ATR): 3028, 2965, 2874, 2811, 1745, 1604, 1571, 1549, 1495, 1455, 1421, 1377, 1282, 1231, 1200, 1125, 1076, 1038, 995, 977, 910, 786, 743 and 699 cm⁻¹. HRMS (ESI) m/z calcd. for C29H38N3O4: 520.2924; found 520.2943 ([M + H]⁺, Δppm 3.7).

General Procedure B for the Esterification of 2-pyridyl-triazolo alcohols

91-105: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to the acid chloride (0.050 mmol). An additional 0.500 mL of pyridine was added to a 1 dram vial and the mixture was heated on a J-KEM heating block (100 °C bottom, 10 °C top) for 18 h. The reaction vessel was cooled to ambient temperature, saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted
with ether (3 × 2 mL). The combined organic layers were dried over MgSO₄ and concentrated. The crude residues were then purified by column chromatography.

91: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: This reaction was performed as described in General Procedure B. 1-(4-chlorophenyl)cyclopentane-1-carbonyl chloride 73 (0.0121 g, 0.050 mmol). The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (4:5) to give 91 (0.0128 g, 97.9%). ¹H NMR (400MHz, CDCl₃) δ 0.83 (d, J = 7.0 Hz, 3H), 1.14 (d, J = 7.0 Hz, 3H), 1.32 (m, 1H), 1.49 (m, 1H), 1.56-1.68 (m, 2H), 1.82-1.93 (m, 5H), 1.99 (m, 1H), 2.56-2.75 (m, 5H), 3.35 (m, 1H), 3.43 (m, 1H), 4.27 (dd, J = 13.2, 7.9 Hz, 1H), 4.59 (dd, J = 14.1, 4.4 Hz, 1H), 4.99 (m, 1H), 7.23(m, 1H), 7.28 (m, 1H), 7.29-7.38 (m, 3H), 7.77 (m, 1H), 8.16 (s, 1H), 8.18 (d, J = 7.9 Hz, 1H) and 8.59 (d, J = 5.3 Hz, 1H), impurity 1.74 (m, 11H); ¹³C (100MHz, CDCl₃) δ 14.25, 20.40, 23.52, 29.44, 29.69, 31.10, 35.78, 36.03, 40.36, 42.26, 53.51, 58.80, 69.36, 75.90, 77.19, 80.53, 120.33, 123.01, 128.15, 128.39, 132.38, 132.80, 137.01, 141.40, 142.06, 147.85, 149.17, 150.30 and 174.75. IR (film-ATR): 2926, 2874, 1719, 1692, 1604, 1571, 1492, 1453, 1421, 1400, 1379, 1309, 1234, 1182, 1120, 1093, 1039, 1014, 966,
829, 785, 760 and 718 cm\(^{-1}\). ESI\(^{+}\)MS \(m/z\) 523 [M + H]\(^+\), HRMS (ESI) \(m/z\) calcd. for 
\(C_{29}H_{36}N_4O_3Cl\): 523.2476; found 523.2475 ([M + H]\(^+\), \(\Delta ppm\) 0.2).

\[ \begin{align*}
\text{OH} & \quad \text{O} \\
69 & \quad \text{N} = \text{N} \\
\text{N} & \quad \text{N} \\
\text{pyridine} & \\
\text{O} & \quad \text{Cl} \\
92 & \quad \text{N} = \text{N}
\end{align*} \]

92: Naphthalen-2-yl [1-{5-[1-{4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl]propan-2-yl] carbonate: This reaction was performed as described in General Procedure B. Naphthalen-2-yl chloroformate 74 (0.0103 g, 0.050 mmol) was the chloride used. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (4:5) to give 92 (0.0061 g, 50.2%).

\(^1\)H NMR (400MHz, CDCl\(_3\)) \(\delta\) 0.88 (d, \(J = 7.0\) Hz, 3H), 1.47 (d, \(J = 6.1\) Hz, 3H), 1.50-1.68 (m, 1H) 1.59 (H\(_2\)O, br s), 1.83 (ddd, \(J = 14.1, 8.8, 4.4\) Hz, 1H), 1.93-1.26 (m, 5H), 3.59 (m, 1H), 4.06 (m, 1H), 4.34 (dd, \(J = 14.1, 7.9\) Hz, 1H), 4.67 (dd, \(J = 14.1, 4.4\) Hz, 1H), 5.10 (m, 1H), 7.20 (m, 1H), 7.37 (d, \(J = 7.9\) Hz, 1H), 7.45 (t, \(J = 7.9\) Hz, 1H), 7.48-7.56 (m, 2H), 7.71-7.77 (m, 2H), 7.86 (m, 1H), 7.97 (m, 1H), 8.14 (s, 1H), 8.16 (s, 1H) and 8.55 (d, \(J = 4.4\) Hz, 1H). IR (film-ATR): 3058, 2960, 2924, 2854, 1757, 1599, 1571, 1509, 1463, 1421, 1379, 1267, 1246, 1223, 1143, 1039, 996, 922, 774 and 746 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for \(C_{28}H_{31}N_4O_4\): 487.2345; found 487.2326 ([M + H]\(^+\), \(\Delta ppm\) 4.0).
93: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 3-(trifluoromethoxy)benzoate: This reaction was performed as described in General Procedure B. 3-(trifluoromethoxy)benzoyl chloride 75 (0.0112 g, 0.050 mmol) was the chloride used. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (3:2) to give 93 (0.0046 g, 36.5%).

$^1$H NMR (400MHz, CDCl$_3$) δ 0.86 (d, J = 6.1 Hz, 3H), 1.41 (d, J = 6.1 Hz, 3H), 1.49-1.64 (m, 1H), 1.61 (H$_2$O, br s), 1.86 (ddd, J = 14.9, 7.9, 4.4 Hz, 1H), 1.93-2.12 (m, 5H), 3.52 (m, 1H), 3.97 (m, 1H), 4.33 (dd, J = 14.1, 7.9 Hz, 1H), 4.64 (dd, J = 13.2, 4.4 Hz, 1H), 5.36 (m, 1H), 7.21 (m, 1H), 7.40 (m, 1H), 7.48 (t, J = 7.9 Hz, 1H), 7.77 (m, 1H), 7.89 (s, 1H), 8.00 (dm, J = 7.9 Hz, 1H), 8.16 (s, 1H), 8.17 (d, J = 7.9 Hz, 1H) and 8.56 (dm, J = 4.4 Hz, 1H). IR (film-ATR): 2935, 1719, 1601, 1594, 1572, 1445, 1421, 1357, 1251, 1216, 1165, 1074, 1039, 996, 964, 908, 785, 760, 708, 678 and 633 cm$^{-1}$. HRMS (ESI) $m/z$ calcd. for C$_{25}$H$_{28}$N$_4$O$_4$F$_3$: 505.2063; found 505.2088 ([M + H]$^+$, Δppm 5.0).
94: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]oxolan-2-yl}propan-2-yl 4-(benzyloxy)benzoate: This reaction was performed as described in General Procedure B. 4-(benzyloxy)benzoyl chloride 76 (0.0123 g, 0.050 mmol) was the chloride used. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (7:3) to give 94 (0.0098 g, 74.4%). $^1$H NMR (400MHz, CDCl$_3$) $\delta$ 0.86 (d, J = 7.0 Hz, 3H), 1.40 (d, H = 6.1 Hz, 3H), 1.50-1.71 (m, 2H), 1.66 (H$_2$O, br s), 1.84 (ddd, J = 14.1, 7.9, 4.4 Hz, 1H), 1.92-2.13 (m, 4H), 3.53 (m, 1H), 3.98 (m, 1H), 4.33 (dd, J = 14.1, 7.9 Hz, 1H), 4.66 (dd, J = 14.1, 4.4 Hz, 1H), 5.33 (m, 1H), 6.70 (d, J = 4.4 Hz, 1H), 7.20 (dd, J = 7.9, 4.4 Hz, 1H), 7.24 (d, J = 3.5 Hz, 1H), 7.50 (t, J = 7.9 Hz, 1H), 7.62 (t, J = 7.9 Hz, 1H), 7.72-7.82 (m, 2H), 8.15 (s, 1H), 8.17 (s, 1H), 8.56 (d, J = 4.4 Hz, 1H). IR (film-ATR): 2961, 2925, 2854, 1715, 1604, 1571, 1531, 1462, 1421, 1356, 1298, 1218, 1151, 1039, 996, 968, 925, 851, 810, 784, 748 and 706 cm$^{-1}$. 

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95: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-acetylpiperidine-4-carboxylate: This reaction was performed as described in General Procedure B. 1-acetylpiperidine-4-carbonyl chloride 77 (0.0095 g, 0.050 mmol) was the chloride used. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (4:1) containing 1% v/v Et₃N to give 95 (0.0073 g, 62.2%). IR (film-ATR): 2931, 2861, 1723, 1640, 1604, 1571, 1446, 1421, 1376, 1314, 1272, 1227, 1180, 1145, 1117, 1062, 1037, 995, 979, 955, 921, 787, 746 and 712 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₅H₃₆N₅O₄: 470.2767; found 470.2784 ([M + H]⁺, Δppm 3.6).

96: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 3-tert-butyl-1-methyl-1H-pyrazole-5-carboxylate: This reaction was performed as described in General Procedure B. 3-t-butyl-methyl-1H-pyrazole-5-carbonyl chloride 78 (0.010 g, 0.050 mmol) was the chloride used. The crude
residue was purified by column chromatography and eluted in EtOAc/Hexanes (4:1) containing 1% v/v Et₃N to give 96 (0.0072 g, 60.8%). ¹H NMR (400MHz, CDCl₃) δ 0.87 (d, J = 6.3 Hz, 3H), 1.30 (s, 9H), 1.38 (d, J = 6.3 Hz, 3H), 1.50-1.66 (m, 2H), 1.70 (H₂O, br s), 1.81 (m, 1H), 1.91-2.13 (m, 4H), 3.52 (m, 1H), 3.96 (m, 1H), 4.12 (s, 3H), 4.35 (dd, J = 14.0, 7.6 Hz, 1H), 4.65 (dd, J = 12.7, 3.8 Hz, 1H), 5.30 (m, 1H), 6.65 (s, 1H), 7.21 (m, 1H), 7.76 (m, 1H), 8.16 (s, 1H), 8.17 (d, J = 7.6 Hz, 1H) and 8.57 (m, 1H); ¹³C (100MHz, CDCl₃) δ 14.30, 20.84, 29.53, 30.46, 31.34, 39.26, 40.44, 42.59, 53.51, 69.58, 76.01, 77.19, 80.75, 107.04, 120.20, 122.68, 122.84, 132.66, 136.82, 148.15, 149.37, 150.44, 159.59 and 160.23. IR (film-ATR): 2962, 2904, 2876, 1714, 1604, 1526, 1461, 1421, 1365, 1256, 1231, 1137, 1087, 1039, 994, 821, 786, 768, 746 and 706 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₆H₃₇N₆O₃: 481.2927; found 481.2947 ([M + H]⁺, Δppm 4.1).

97: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 5-chloro-1-methyl-1H-pyrazole-4-carboxylate: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to 5-chloro-1-methyl-1H-pyrazole-4-carbonyl chloride 79 (0.0119 g, 0.0664 mmol). Pyridine (0.250 mL, 0.2458 mmol) was added and the mixture was heated on the reaction block (100 °C bottom, 10 °C top) for 18 h. The mixture was purified by column chromatography and
eluted in EtOAc/Hexanes (1:1) containing 1% v/v Et₃N to give 97 (0.0058 g, 50.7%). IR (fil-ATR): 3140, 2971, 2875, 1710, 1604, 1571, 1538, 1471, 1420, 1275, 1227, 1168, 1114, 1035, 995, 980, 909, 831, 786, 774, 746, 719, 645, 621 and 556 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₂H₂₈N₆O₅Cl: 459.1911; found 459.1890 ([M + H]⁺, Δppm 4.7).

98: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 4-(trifluoromethoxy)benzoate: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to 4-(trifluoromethoxy)benzoyl chloride 80 (0.0112 g, 0.050 mmol). Pyridine (0.250 mL, 2.458 mmol) was added and the mixture was heated on the reaction block (100 °C bottom, 10 °C top) for 15 min. The mixture was cooled to ambient temperature and purified by column chromatography and eluted in EtOAc/Hexanes (2:3) containing 1% v/v Et₃N to give 98 (0.0084 g, 66.6%). IR (film-ATR): 3064, 2972, 2877, 1717, 1605, 1572, 1549, 1505, 1472, 1421, 1358, 1251, 1220, 1163, 1102, 1039, 1018, 996, 977, 924, 856, 785, 770, 745, 709, 659, 621, 571 and 535 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₅H₂₈N₄O₄F₃: 505.2063; found 505.2084 ([M + H]⁺, Δppm 4.2).
99: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]oxolan-2-yl}propan-2-yl) 5-tert-butyl-2-methylfuran-3-carboxylate: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to 5-tert-butyl-2-methylfuran-3-carbonyl chloride 81 (0.010 g, 0.050 mmol). Pyridine (0.250 mL, 0.2548 mmol) was added and the mixture was heated on the reaction block (115 °C bottom, 10 °C top) for 18 h. The mixture was then purified by column chromatography and eluted in EtOAc/Hexanes (2:3) containing 1% v/v Et₃N to give 99 (0.0091 g, 75.7%). IR (film-ATR): 3146, 3054, 2967, 2905, 2872, 1707, 1605, 1577, 1550, 1461, 1420, 1397, 1364, 1297, 1254, 1231, 1207, 1155, 1117, 1063, 1039, 995, 978, 942, 816, 778, 745, 712, 665, 621, 585 and 539 cm⁻¹. HRMS (ESI) m/z calcd. for C₂₇H₃₇N₄O₄: 481.2815; found 481.2802 ([M + H]⁺, Δppm 2.7).

100: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]oxolan-2-yl}propan-2-yl) 2H-1,3-benzodioxole-5-carboxylate: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to 2H-1,3-benzodioxole-5-carbonyl chloride 82
(0.010 g, 0.0541 mmol). Pyridine (0.250 mL, 0.2548 mmol) was added and the mixture was heated on the heating block (115 °C bottom, 10 °C top) for 16 h. The mixture was then cooled to ambient temperature and purified by column chromatography and eluted in EtOAc/Hexanes (1:1) containing 1% v/v Et$_3$N to give 100 (0.0079 g, 68.0%). IR (film-ATR): 3138, 3084, 2971, 2932, 1705, 1604, 1571, 1549, 1504, 1489, 1441, 1421, 1368, 1278, 1256, 1231, 1204, 1160, 1104, 1071, 1035, 996, 977, 931, 824, 806, 785, 762, 746, 720, 668, 621, 579 and 544 cm$^{-1}$.

![Chemical Structure](image)

101: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl furan-2-carboxylate: A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to furan-2-carbonyl chloride 83 (0.0065 g, 0.050 mmol). Pyridine (0.250 mL, 0.2458 mmol) was added and the mixture was heated on the heating block (115 °C bottom, 10 °C top) for 18 h. The mixture was then cooled to ambient temperature and purified by column chromatography and eluted in EtOAc/Hexanes (3:2) containing 1% v/v Et$_3$N to give 101 (0.0088 g, 85.8%). IR (film-ATR): 3141, 2971, 2876, 1712, 1679, 1604, 1580, 1571, 1549, 1473, 1421, 1395, 1297, 1230, 1181, 1117, 1076, 1040, 1013, 996, 977, 939, 885, 786, 764, 712 and 619 cm$^{-1}$.
**102: 1-(5-1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl)oxolan-2-yl]propan-2-yl 3-(methylsulfanyl)propanoate:** A 1.0 M solution of 69 (0.025 mL, 0.025 mmol) in pyridine was added to 3-(methylsulfonyl)propanoyl chloride 84 (0.0069 g, 0.050 mmol). Pyridine (0.250 mL, 0.2458 mmol) was added and the mixture was heated on the heating block (115 °C bottom, 10 °C top) for 18 h. The mixture was cooled to ambient temperature then purified by column chromatography and eluted in EtOAc/Hexanes (3:2) containing 1% v/v Et$_3$N to give 102 (0.0103 g, 98.4%). IR (film-ATR): 3142, 3052, 2970, 2916, 2875, 1727, 1604, 1571, 1549, 1472, 1421, 1370, 1246, 1203, 1141, 1039, 995, 977, 786, 746, 712 and 621 cm$^{-1}$. HRMS (ESI) $m/z$ calcd. for C$_{22}$H$_{33}$N$_5$O$_3$S: 419.2117; found 419.2084 ([M + H]$^+$, Δppm 7.8).

**147: N-(3-ethynylphenyl)acetamide:** Acetic anhydride (11.88 mL, 125.6 mmol) was added dropwise to a stirred solution of 3-ethynyl aniline (6.57 mL, 62.82 mmol) in CH$_2$Cl$_2$ (63 mL) chilled on an ice bath to 0 °C. When no more gas evolved, the ice bath was removed and the solution was stirred at ambient temperature for 2 h. Saturated aqueous NaHCO$_3$ (10 mL) was added and the mixture was extracted...
with ether (3 × 10 mL). Combined organic layers were dried over MgSO₄ and concentrated. The crude solid was re-crystallized from CH₂Cl₂ to give 3-ethynyl acetanilide 147 (5.00 g, 50.0%) as a pale yellow solid.

148: N-(4-ethynylphenyl)acetamide: A 1.0 M solution of 4-ethynyl aniline (2.00 g, 17.1 mmol) in CH₂Cl₂ was cooled on an ice bath to 0 °C. Acetic anhydride (3.23 mL, 34.1 mmol) was added dropwise over 5 min and the solution was stirred at ambient temperature for 2 h. The solution was washed with saturated aqueous NaHCO₃ (3 × 5 mL), water (5 mL), dried over MgSO₄ and concentrated to give a pale yellow solid, which was re-crystallized from CH₂Cl₂ to give 148 (1.67 g, 61.4%).

151: 1-(4-methylphenyl)cyclopentane-1-carbonyl chloride: 1-(4-methylphenyl)cyclopentane-1-carboxylic acid (0.100 g, 0.4895 mmol) was added to a conical vial which was then briefly flame dried and flushed with argon. When cooled to ambient temperature, a solution of benzene/CH₂Cl₂ (5:2, 1.4
mL) and a drop of DMF were added. The suspension was cooled to 0 °C on an ice bath for 5 min and oxalyl chloride (0.0512 mL, 0.5874 mmol) was added dropwise, briefly evolving gas. After the gas bubbling subsided, the ice bath was removed and the mixture continued to stir at ambient temperature. Gentle bubbling began again and the solid acid began to disappear. When the bubbling ceased and the acid had been consumed, the solution was transferred to a scintillation vial and the solvent was evaporated at ambient temperature under gentle vacuum to give 151 in quantitative yield as an oily residue was subsequently used without further purification.

152: 1-phenylcyclopentane-1-carbonyl chloride: 1-Phenylcyclopentane-1-carboxylic acid (0.100 g, 0.5256 mmol) was taken up in a solution of benzene/CH₂Cl₂ (5:2, 1.4 mL) and 1 drop of DMF was added. This solution was cooled to 0 °C on an ice bath and oxalyl chloride (0.055 mL, 0.0801 g, 0.6037 mmol) was added dropwise. When no more gas evolved, the solution was concentrated by rotary evaporation and then placed under high vac for 30 min to give 1-phenylcyclopentane-1-carbonyl chloride 152 in quantitative yield as a clear oil which was subsequently used without further purification.
154: 1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl chlorophenyl)cyclopentane-1-carboxylate: Azidoalcohol 8 (0.0651 g, 0.3052 mmol) as a solution in benzene (~1.0 mL) was added to activated zinc (0.184 g, 2.81 mmol) and stirred rapidly enough to keep the zinc suspended. 1-(4-Chlorophenyl)cyclopentane-1-carbonyl chloride 150 (0.1113 g, 0.4578 mmol) was added as a solution in benzene (~1.0 mL). This suspension continued to stir at ambient temperature for 18 h. The next morning, the reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1) so more chloride (0.025 g, 0.0061 mmol) and zinc (0.020 g, 1.31 mmol) were added and the reaction was stirred for a further 24 h. Upon completion, the suspension was filtered through a cotton plug which was then rinsed with benzene (4 × 2 mL) The solvent was evaporated to give a crude residue which was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 154 (0.1127 g, 87.9%) as a white solid. ESI+MS m/z 392 [M + H]+.

155: 1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl methylphenyl)cyclopentane-1-carboxylate: A solution of chloride 151 (0.109 g, 153
0.4894 mmol) in benzene (≈1.0 mL) was added to a solution of 8 (0.0300 g, 0.1406 mmol) in benzene (≈1.0 mL). Activated zinc (0.046 g, 0.703 mmol) was added and the mixture was stirred at ambient temperature for 3 d fast enough to keep the zinc suspended. The suspension was filtered through a cotton plug which was then washed with benzene (2 mL) and ether (2 mL). The resulting solution was then washed with saturated aqueous NaHCO₃ (2 mL) then dried over MgSO₄ and concentrated to give a crude, off white solid which was then purified by flash chromatography and eluted in EtOAc/Hexanes (1:9) to give 155 (0.0572 g, >99.9%). ESI+MS m/z 372 [M + H]+.

![Chemical Structure]

156: 1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl 1-phenylcyclopentane-1-carboxylate: 1-Phenylcyclopentane-1-carbonyl chloride 152 (0.1097 g, 0.5231 mmol) was added to 8 (0.0308 g, 0.1406 mmol) as a solution in benzene (0.500 mL). Activated zinc (0.046 g, 0.703 mmol) was then added and the mixture was stirred at ambient temperature for 20 h. The mixture was filtered through a cotton plug. The residue was then purified by column chromatography and eluted in EtOAc/Hexanes (1:9) to give 156 in quantitative yield. IR (film-ATR): 3059, 3027, 2971, 2874, 2097, 1792, 1722, 1600, 1494, 1448, 1380, 1300, 1249,
1185, 1166, 1124, 1056, 1028, 983, 731, 697, 623, 583, 554 cm⁻¹. ESI⁺MS m/z 358 [M – N₂ + H]⁺.

157: 1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl 2-(4-chlorophenyl)acetate: 2-(4-Chlorophenyl)acetyl chloride 153 (0.0532 g, 0.2813 mmol) was added to 8 (0.030 g, 0.1407 mmol) as a solution in benzene (0.500 mL). Activated zinc (0.046 g, 0.7035 mmol) was added and the mixture was stirred at ambient temperature fast enough to keep the zinc suspended. After 1 h, the mixture was filtered through a cotton plug which was then rinsed with benzene. The solution was concentrated and the residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:9) to give 157 (0.0319 g, 61.9%). IR (film-ATR): 2970, 2877, 2096, 1732, 1493, 1460, 1381, 1255, 1220, 1090, 1017, 961, 870, 808, 763, 629, 584 and 529 cm⁻¹. ESI⁺MS m/z 338 [M – N₂ + H]⁺.
158: 1-{5-[1-(4-phenyl-1H-1,2,3-triazol-1-yl)propan-2-yl]oxolan-2-yl}propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: A 0.5 M solution of 154 (0.0428 mL, 0.009 g, 0.0214 mmol) in EtOH/H₂O (5:1) was mixed with a 0.5 M solution of ethynylbenzene 144 (0.856 mL, 0.0044 g, 0.0428 mmol) in EtOH/H₂O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0064 mL, 0.0013 g, 0.0071 mmol) and a 0.3 M aqueous solution of CuSO₄·5H₂O (0.0143 mL, 0.0007 g, 0.0043 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (3 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:6) to give 158 (=0.0045 g, 40.2%). IR (film-ATR): 3128, 3063, 2968, 2874, 1717, 1611, 1492, 1462, 1379, 1300, 1229, 1184, 1166, 1122, 1093, 1062, 1043, 1014, 971, 831, 765, 696, 620, 581, 572, 559 and 539 cm⁻¹. ESI⁺MS m/z 522 [M + H]⁺.
**159: 1-(5-{1-[4-(4-chlorophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate:** A 0.5 M solution of 154 (0.0428 mL, 0.009 g, 0.0214 mmol) in EtOH/H₂O (5:1) was mixed with a 0.5 M solution of 1-chloro-4-ethynyl benzene 145 (0.0856 mL, 0.0058 g, 0.0428 mmol) in EtOH/H₂O (5:1). Then a 1.1 M aqueous solution of ascorbate (0.0064 mL, 0.0013 g, 0.0071 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0143 mL, 0.0007 g, 0.0043 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (1 mL) was added and the mixture was extracted with EtOAc (3 × 2 mL). The combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography to give 159 (0.0052 g, 43.7%). IR (film-ATR): 3129, 2959, 2874, 1716, 1551, 1485, 1455, 1401, 1380, 1355, 1231, 1185, 1166, 1122, 1092, 1063, 1041, 1014, 971, 832, 762, 720, 669, 604, 596, 562, 536 and 526 cm⁻¹.

¹. ESI⁺MS m/z 556 [M + H]⁺.
160: 1-(5-{1-[4-(4-methylphenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: A 0.5 M solution of 154 (0.043 mL, 0.0091 g, 0.0216 mmol) in EtOH/H₂O (5:1) was mixed with a 0.5 M solution of 4-ethynyltoluene 146 (0.086 mL, 0.005 g, 0.0432 mmol) in EtOH/H₂O (5:1) and additional EtOH/H₂O (5:1, 0.200 mL) was added. Then a 1.1 M aqueous solution of ascorbate (0.013 mL, 0.0025 g, 0.0144 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.029 mL, 0.0013 g, 0.0086 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL) and washed with water (3 × 3 mL). The combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:3) to give 160 (0.0039 g, 33.6%). ESI^+MS m/z 536 [M + H]^+.
161: 1-(5-{1-[4-(3-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: A 0.5 M solution of 154 (0.0358 mL, 0.0075 g, 0.0179 mmol) in EtOH/H2O (5:1) was mixed with a 0.5 M solution of 3-ethynyl acetonilide 147 (0.0714 mL, 0.0056 g, 0.0357 mmol) in EtOH/H2O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0033 mL, 0.0018 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0060 mL, 0.0003 g, 0.0018 mmol) were added and the cloudy, yellow mixture was stirred at ambient temperature for 12 h. The next day, the reaction was incomplete as determined by TLC (100% EtOAc) and more ascorbate (0.006 mL, 0.0012 g, 0.0066 mmol) and CuSO₄•5H₂O (0.048 mL, 0.023 g, 0.0144 mmol) were added and the reaction mixture was stirred for an additional 24 h. The crude mixture was purified directly by column chromatography and eluted in EtOAc/Hexanes (2:3) to give 161 (0.0015 g, 14.4%). $^{13}$C (100MHz, CDCl₃) δ 14.73, 20.47, 23.45, 23.49, 24.71, 29.72, 31.06, 35.63, 35.77, 40.34, 40.99, 42.01, 53.31, 58.90, 69.55, 75.73, 80.49, 116.57, 119.38, 121.38, 121.45, 128.22, 128.26, 129.66, 132.49, 138.45, 141.88, 168.32, 170.62. HRMS (ESI) m/z calcd. for C₃₂H₄₀N₄O₄Cl: 579.2738; found 579.2753 ([M + H]$^+$, Δppm 2.6)
162: 1-(5-[[4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: A 0.5 M solution of 154 (0.0358 mL, 0.0075 g, 0.0179 mmol) in EtOH/H2O (5:1) was mixed with a 0.5 M solution of 4-ethynyl acetanilide 148 (0.0714 mL, 0.0056 g, 0.0357 mmol) in EtOH/H2O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0033 mL, 0.0018 mmol) and a 0.3 M aqueous solution of CuSO4•5H2O (0.0060 mL, 0.0003 g, 0.0018 mmol) were added and the cloudy, yellow mixture was stirred at ambient temperature for 12 h. The next day, the reaction was incomplete as determined by TLC (100% EtOAc) and more ascorbate (0.006 mL, 0.0012 g, 0.0066 mmol) and CuSO4•5H2O (0.048 mL, 0.023 g, 0.0144 mmol) were added and the reaction mixture was stirred for an additional 24 h. The crude mixture was purified directly by column chromatography and eluted in EtOAc/Hexanes (2:3) to give 162 (0.0094 g, 90.4%). $^1$H NMR (400MHz, CDCl$_3$) δ 0.87 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.4 Hz, 3H), 1.31 (m, 1H), 1.49 (m, 1H), 1.53-1.62 (m, 2H), 1.66 (m, 1H), 1.70-1.84 (m, 6H), 1.85-1.98 (m, 2H), 2.20 (s, 3H), 2.61 (m, 1H), 2.70 (m, 1H), 3.24 (m, 1H), 3.34 (m, 1H), 4.35 (dd, J = 13.7, 6.8 Hz, 1H), 4.50 (dd, J = 13.7, 3.9 Hz, 1H), 5.09 (m, 1H), 7.22 (dm, J = 8.8 Hz, 2H), 7.27 (dm, J = 8.8 Hz, 2H), 7.58 (dm, J = 8.8 Hz, 2H), 7.86 (s, 1H), 7.87 (dm, J = 8.8 Hz, 2H); $^{13}$C (100MHz, CDCl$_3$) δ 14.50, 20.49, 23.48, 23.53, 24.72, 29.61, 31.06, 35.76, 35.81, 40.34, 160
42.23, 53.21, 58.86, 69.11, 75.68, 80.32, 119.84, 120.73, 126.40, 126.96, 128.17, 128.35, 128.42, 132.41, 137.57, 141.99, 146.96, 168.18, 174.88. HRMS (ESI) m/z calcd. for C₃₂H₄₅N₄O₄Cl: 579.2738; found 579.2795 ([M + H]⁺, Δppm 9.8). ESI⁺MS m/z 579 (M + H)⁺.

163: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-methylphenyl)cyclopentane-1-carboxylate: Azide 155 (0.0087 g, 0.0217 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and 0.5 M solution of 2-ethynyl pyridine 143 (0.0868 mL, 0.0045 g, 0.0434 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0434 mL 0.0084 g, 0.0477 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0796 mL, 0.0038 g, 0.0238 mmol). This mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 163 (0.0023 g, 21.1%). ¹H NMR (500MHz, CDCl₃) δ 0.84 (d, J = 6.8
Hz, 3H), 1.15 (d, J = 6.4 Hz, 3H), 1.26 (grease, br s), 1.32 (m, 1H), 1.48 (m, 1H), 1.56 (H$_2$O, br s), 1.58-1.68 (m, 3H), 1.68-1.78 (m, 4H), 1.80-1.89 (m, 2H), 1.92 (m, 1H), 2.00 (m, 1H), 2.30 (s, 3H), 2.60 (m, 1H), 2.70 (m, 1H), 3.35 (m, 1H), 3.48 (m, 1H), 4.29 (dd, J = 13.7, 7.8 Hz, 1H), 4.60 (dd, J = 13.7, 3.9 Hz, 1H), 5.00 (m, 1H), 7.09 (d, J = 7.8 Hz, 2H), 7.22 (ddd, J = 7.3, 4.9, 0.98 Hz, 1H), 7.27 (d, J = 7.8 Hz, 2H), 7.77 (dt, J = 7.8, 2.0 Hz, 1H), 8.15 (s, 1H), 8.18 (dm, J = 7.8 Hz, 1H), 8.59 (dm, J = 4.9 Hz, 1H). ESI$^+$MS m/z 503 [M + H]$^+$. 

\[ \text{164: 1-(5-\{(4-aminophenyl)-1H-1,2,3-triazol-1-yl\)propan-2-yl\)oxolan-2-yl\)propan-2-yl 1-(4-methylphenyl)cyclopentane-1-carboxylate: Azide 155 (0.0087 g, 0.0217 mmol) was taken up in EtOH/H$_2$O (5:1, 0.200 mL) and a 0.5 M solution of 3-ethynyl acetanilide 147 (0.0868 mL, 0.0069 g, 0.0434 mmol) in EtOH/H$_2$O was added, followed by a 1.1 M aqueous solution of ascorbate (0.0118 mL, 0.0023 g, 0.0130 mmol) and a 0.3 M aqueous solution of CuSO$_4$•5H$_2$O (0.0216 mL, 0.0010 g, 0.0065 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO$_3$ (2 mL) was added and the mixture was extracted with EtOAc (3 x 3 mL). Combined organic layers were washed with water (2 x 2 mL) and brine (2 mL) then dried} \]
over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 164 (0.0036 g, 29.8%). ¹H NMR (500MHz, CDCl₃) δ 0.90 (d, J = 6.8 Hz, 3H), 1.15 (d, J = 6.4 Hz, 3H), 1.26 (grease, br s), 1.29 (m, 1H), 1.47 (m, 1H), 1.54-1.60 (m, 2H), 1.61-1.78 (m, 5H), 1.78-1.86 (m, 2H), 1.89-1.97 (m, 1H), 2.18 (s, 3H), 2.26 (s, 3H), 2.57 (m, 1H), 2.66 (m, 1H), 3.15 (m, 1H), 3.30 (m, 1H), 4.38 (dd, J = 13.7, 5.8 Hz, 1H), 4.46 (dd, J = 13.7, 3.9 Hz, 1H), 5.03 (m, 1H), 7.01 (d, J = 7.8 Hz, 2H), 7.19 (dm, J = 8.3 Hz, 2H), 7.40 (t, J = 7.8 Hz, 1H), 7.57 (br s, 1H), 7.70-7.73 (m, 2H), 7.83 (dm, J = 7.8 Hz, 1H), 7.86 (s, 1H). ESI’MS m/z 559 [M + H]⁺.

165: 1-(5-{1-[4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-methylphenyl)cyclopentane-1-carboxylate: Azide 155 (0.0087 g, 0.0217 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and a 0.5 M solution of 4-ethynyl acetonilide 148 (0.0868 mL, 0.0069 g, 0.0434 mmol) in EtOH/H₂O was added, followed by a 1.1 M aqueous solution of ascorbate (0.0118 mL, 0.0023 g, 0.0130 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0216 mL, 0.0010 g, 0.0065 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL)
was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 165 (0.0038 g, 31.4%). ³H NMR (500MHz, CDCl₃) δ 0.87 (d, J = 6.8 Hz, 3H), 1.14 (d, J = 6.4 Hz, 3H), 1.26 (grease, br s), 1.30 (m, 1H), 1.47 (m, 1H), 1.53-1.60 (m, 2H), 1.64-1.78 (m, 5H), 1.78-1.87 (m, 2H), 1.88-1.97 (m, 2H), 2.20 (s, 3H), 2.28 (s, 3H), 2.61 (m, 1H), 2.70 (m, 1H), 3.23 (m, 1H), 3.36 (m, 1H), 4.35 (dd, J = 13.7, 6.4 Hz, 1H), 4.50 (dd, J = 13.7, 3.9 Hz, 1H), 5.09 (m, 1H), 7.05 (d, J = 7.3 Hz, 2H), 7.21 (s, 1H), 7.23 (d, J = 7.3 Hz, 2H), 7.58 (d, J = 8.8 Hz, 2H), 7.86 (s, 1H), 7.88 (d, J = 8.8 Hz, 2H).

166: 1-(5-{1-[4-(4-nitrophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-methylphenyl)cyclopentane-1-carboxylate: Azide 155 (0.0087 g, 0.0217 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and a 0.5 M solution of 4-ethynyl nitrobenzene 149 (0.0880 mL, 0.0065 g, 0.0440 mmol) in EtOH/H₂O was added, followed by a 1.1 M aqueous solution of ascorbate (0.0120 mL, 0.0023 g, 0.0132 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0220
mL, 0.0011 g, 0.0066 mmol) were added and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 166 (0.0041 g, 34.2%). $^1$H NMR (500MHz, CDCl₃) δ 0.92 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.4 Hz, 3H), 1.29 (m, 1H), 1.44-1.54 (m, 2H), 1.62-1.72 (m, 3H), 1.71-1.80 (m, 3H), 1.80-1.87 (m, 2H), 1.87-1.98 (m, 2H), 2.28 (s, 3H), 2.61 (m, 1H), 2.69 (m, 1H), 3.14 (m, 1H), 3.26 (m, 1H), 4.45-4.53 (m, 2H), 5.19 (m, 1H), 7.04 (d, J = 8.8 Hz, 2H), 7.21 (dm, J = 8.3 Hz, 2H), 8.17 (dm, J = 8.8 Hz, 2H), 8.18 (s, 1H), 8.28 (dm, J = 8.8 Hz, 2H). ESI$^+$MS m/z 547 [M + H]$^+$.

167: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g, 0.0192 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL). A 0.5 M solution of 2-ethynyl pyridine 143 (0.115 mL, 0.0059 g, 0.0576 mmol) in EtOH/H₂O (5:1) was then added followed by a 1.1 M aqueous solution of ascorbate (0.0279 mL, 0.0049 g, 0.0307 mmol) and a 0.3 M aqueous solution of CuSO₄·5H₂O (0.070 mL, 0.0034 g, 0.0211
mmol). This mixture was stirred at ambient temperature for 3 d. Saturated aqueous NaHCO₃ (2 mL) was added and the suspension was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 × 2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 167 (0.0018 g, 19.1%). ¹H NMR (500MHz, CDCl₃) δ 0.83 (d, J = 6.4 Hz, 3H), 1.15 (d, J = 6.4 Hz, 3H), 1.27-1.34 (m, 2H), 1.43-1.51 (m, 2H), 1.58-1.80 (m, 5H), 1.56 (H₂O, br s), 1.80-1.90 (m, 2H), 1.91-2.03 (m, 2H), 2.62 (m, 1H), 2.73 (m, 1H), 3.34 (m, 1H), 3.45 (m, 1H), 4.29 (dd, J = 13.7, 7.8 Hz, 1H), 4.60 (dd, J = 13.7, 4.5 Hz, 1H), 5.01 (m, 1H), 7.17-7.24 (m, 2H), 7.27-7.31 (m, 2H), 7.37-7.41 (m, 2H), 7.77 (dt, J = 7.8, 2.0 Hz, 1H), 8.15 (s, 1H), 8.18 (dm, J = 7.8 Hz, 1H) and 8.59 (dm, J = 4.9 Hz, 1H). ESI⁺MS m/z 489 [M + H]⁺.

168: 1-(5-{1-[4-(4-chlorophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g, 0.0192 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and a 0.5 M solution of 4-chloro-1-ethynyl benzene 145 (0.115 mL, 0.0079 g, 0.0576 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0058 mL,
0.0011 g, 0.0064 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0126 mL, 0.0006 g, 0.0038 mmol). This mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 168 (0.0018 g, 18.0%). ^1H NMR (500MHz, CDCl₃) δ 0.88 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.4 Hz, 3H), 1.26-1.32 (m, 2H), 1.46 (m, 1H), 1.54 (ddd, J = 14.2, 9.3, 3.4 Hz, 1H), 1.63-1.70 (m, 3H), 1.72-1.80 (m, 3H), 1.80-1.87 (m, 2H), 1.88-1.99 (m, 2H), 2.62 (m, 1H), 2.71 (m, 1H), 3.19 (m, 1H), 3.29 (m, 1H), 4.40 (dd, J = 13.7, 5.9 Hz, 1H), 4.49 (dd, J = 13.7, 3.9 Hz, 1H), 5.13 (m, 1H), 7.16-7.20 (m, 1H), 7.23 (m, 1H), 7.31-7.35 (m, 2H), 7.39 (dm, J = 8.8 Hz, 2H), 7.89 (dm, J = 8.8 Hz, 2H) and 7.93 (s, 1H). ESI^+MS m/z 522 [M + H]^+.

169: 1-(5-{1-[4-(4-methylphenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g, 0.0192 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and a 0.5 M solution of 4-ethynyl toluene 146 (0.115 mL, 0.0067 g, 0.0576 mmol) in EtOH/H₂O (5:1) was
added, followed by a 1.1 M aqueous solution of ascorbate (0.0021 mL, 0.0041 g, 
0.0230 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.064 mL, 0.0031 g, 
0.0192 mmol). This mixture was stirred at ambient temperature for 18 h. 
Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted 
with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 
ml) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude 
residue was purified by column chromatography and eluted in EtOAc/Hexanes 
(1:4) to give 169 (0.0024 g, 25.0%). ¹H NMR (500MHz, CDCl₃) δ 0.86 (d, J = 6.8 
Hz, 3H), 1.14 (d, J = 6.4 Hz, 3H), 1.25 (grease), 1.29 (m, 1H), 1.46 (m, 1H), 1.57 
(ddd, J = 14.2, 8.8, 3.9 Hz, 1H), 1.58 (H₂O, br s), 1.63-1.72 (m, 3H), 1.73-1.80 (m, 
3H), 1.79-1.88 (m, 2H), 1.89-1.98 (m, 2H), 2.38 (s, 3H), 2.63 (m, 1H), 2.73 (m, 1H), 
3.23 (m, 1H), 3.35 (m, 1H), 4.34 (dd, J = 13.7, 6.8 Hz, 1H), 4.51 (dd, J = 13.7, 3.9 
Hz, 1H), 5.09 (m, 1H), 7.18 (m, 1H), 7.22-7.25 (m, 3H), 7.29-7.38 (m, 3H), 7.80 (d, 
J = 7.8 Hz, 2H) and 7.84 (s, 1H). ESI⁺MS m/z 502 [M + H]⁺.

![Chemical Structure](image)

**170**: 1-(5-{1-[4-{3-acetamidophenyl}-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-
yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g, 
0.0192 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL). A 0.5 M solution of 3-
ethynyl acetonilide 147 (0.1152 mL, 0.0092 g, 0.0576 mmol) in EtOH/H$_2$O (5:1) was then added followed by a 1.1 M aqueous solution of ascorbate (0.0058 mL, 0.0011 g, 0.0064 mmol) and a 0.3 M aqueous solution of CuSO$_4$·5H$_2$O (0.0126 mL, 0.0006 g, 0.0038 mmol). This mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO$_3$ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO$_4$ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:1) to give 170 (0.0022 g, 21.0%). $^1$H NMR (500 MHz, CDCl$_3$) δ 0.89 (d, $J$ = 6.4 Hz, 3H), 1.14 (d, $J$ = 6.4 Hz, 3H), 1.29 (m, 1H), 1.46 (m, 1H), 1.57 (m, 1H), 1.64-1.78 (m, 6H), 1.80-1.88 (m, 2H), 1.88-2.00 (m, 2H), 2.18 (s, 3H), 2.60 (m, 1H), 2.69 (m, 1H), 3.18 (m, 1H), 3.30 (m, 1H), 4.37 (dd, $J$ = 13.7, 6.4 Hz, 1H), 4.47 (dd, $J$ = 13.7, 3.9 Hz, 1H), 5.05 (m, 1H), 7.17 (m, 1H), 7.20-7.25 (m, 2H), 7.30-7.33 (m, 2H), 7.40 (t, $J$ = 7.8 Hz, 1H), 7.50 (br s, 1H), 7.70 (dm, $J$ = 7.8 Hz, 1H), 7.73 (s, 1H), 7.80 (dm, $J$ = 8.3 Hz, 1H), 7.86 (s, 1H). ESI$^+$MS m/z 545 [M + H]$^+$. 

171: 1-(5-{1-[4-{4-acetamidophenyl]-1H-1,2,3-triazol-1-yl}propan-2-yl}oxolan-2-yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g,
0.0192 mmol) was taken up in EtOH/H$_2$O (5:1, 0.200 mL) and a 0.5 M solution of 4-ethynyl acetanilide 148 (0.0768 mL, 0.0061 g, 0.0384 mmol) in EtOH/H$_2$O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0104 mL, 0.002 g, 0.0115 mmol) and a 0.3 M aqueous solution of CuSO$_4$$\cdot$5H$_2$O (0.0193 mL, 0.0009 g, 0.0058 mmol). This mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO$_3$ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO$_4$ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 171 (0.0025 g, 23.4%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.86 (d, J = 6.8 Hz, 3H), 1.14 (d, J = 6.4 Hz, 3H), 1.25 (grease, br s), 1.28 (m, 1H), 1.46 (m, 1H), 1.55 (H$_2$O, br s), 1.56 (m, 1H), 1.62-1.80 (m, 7H), 1.80-1.89 (m, 2H), 1.89-1.98 (m, 2H), 2.20 (s, 3H), 2.63 (m, 1H), 2.72 (m, 1H), 3.23 (m, 1H), 3.34 (m, 1H), 4.35 (dd, J = 13.7, 6.4 Hz, 1H), 4.50 (dd, J = 13.7, 3.9 Hz, 1H), 5.11 (m, 1H), 7.16-7.22, (m, 2H), 7.24 (m, 1H), 7.32-7.36 (m, 2H), 7.58 (d, J = 8.8 Hz, 2H), 7.86 (s, 1H), 7.88 (d, J = 8.8 Hz, 2H). ESI$^+$MS m/z 545 [M + H]$^+$. 
172: 1-(5-{1-[4-(4-nitrophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-phenylcyclopentane-1-carboxylate: Azide 156 (0.0074 g, 0.0192 mmol) was taken up in EtOH/H$_2$O (5:1, 0.200 mL) and a 0.5 M solution of 4-ethynyl nitrobenzene 149 (0.0768 mL, 0.0056 g, 0.0384 mmol) in EtOH/H$_2$O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0104 mL, 0.002 g, 0.0115 mmol) and a 0.3 M aqueous solution of CuSO$_4$$\cdot$5H$_2$O (0.0193 mL, 0.0009 g, 0.0058 mmol). This mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO$_3$ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO$_4$ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 172 (0.0024 g, 28.4%). $^1$H NMR (500MHz, CDCl$_3$) δ 0.91 (d, 6.8 Hz, 3H), 1.13 (dd, J = 6.4 Hz, 3H), 1.29 (m, 1H), 1.43-1.55 (m, 3H), 1.55 (H$_2$O, br s), 1.64-1.71 (m, 3H), 1.73-1.82 (m, 3H), 1.82-2.00 (m, 3H), 2.63 (m, 1H), 2.73 (m, 1H), 3.14 (m, 1H), 3.24 (m, 1H), 4.49 (d, J = 4.4 Hz, 2H), 5.19 (m, 1H), 7.18 (m, 1H), 7.22-7.25 (m, 2H), 7.32-7.34 (m, 2H), 8.17 (dm, J = 8.8 Hz, 2H), 8.18 (s, 1H) and 8.28 (dm, J = 8.8 Hz, 2H). ESI$^+$MS m/z 533 [M + H]$^+$. 

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173: 1-(5-{1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 2-(4-chlorophenyl)acetate: A 0.5 M solution of 69 (0.062 mL, 0.0097 g, 0.031 mmol) in benzene was mixed with a 0.5 M solution of 4-chlorophenyl acetyl chloride 153 (0.068 mL, 0.0063 g, 0037 mmol) in benzene. Activated zinc (0.0022 g, 0.0337 mmol) was added and the reaction was stirred at ambient temperature for 30 min. The reaction remained incomplete as determined by TLC (100% EtOAc) so more chloride 153 (0.068 mL, 0.0063 g, 0.0337 mmol) was added and the mixture was stirred for an additional 30 min. The solvent was transferred to a clean 1 dram vial and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc containing 1% Et₃N to give 173 (0.0099 g, 68.8%). IR (film-ATR): 3145, 3053, 2972, 2876, 1729, 1604, 1571, 1549, 1492, 1472, 1421, 1358, 1253, 1223, 1143, 1090, 1040, 1016, 995, 961, 785, 745, 712, 622, 582, 573, 538 cm⁻¹. ESI⁺MS m/z 469 [M + H]⁺.

174: 1-(5-{1-[4-(4-chlorophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 2-(4-chlorophenyl)acetate: Azide 157 (0.0054 g, 0.0148 mmol)
was taken up in EtOH/H₂O (5:1, 0.200 mL). 4-chloroethylbenzyl 145 (0.0888 mL, 0.0061 g, 0.0444 mmol) was added as a solution in EtOH/H₂O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0045 mL, 0.0049 mmol) and 0.3 M aqueous solution of CuSO₄•5H₂O (0.010 mL, 0.002 mmol) were added and the mixture was stirred at ambient temperature for 18 h. The next day, the mixture was purified directly by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 174 (0.0034 g, 45.9%). ³¹H NMR (500 MHz, CDCl₃) δ 0.90 (d, J = 6.4 Hz, 3H), 1.24 (d, J = 6.4, 3H), 1.44 (m, 1H), 1.51-1.61 (m, 4H), 1.65 (ddd, J = 14.2, 9.3, 3.9 Hz, 1H), 1.79 (ddd, J = 14.2, 9.3, 3.9 Hz, 1H), 1.85-2.04 (m, 3H), 3.34 (m, 1H), 3.57 (s, 2H), 3.67 (m, 1H), 4.38 (dd, J = 13.7, 6.8 Hz, 1H), 4.53 (dd, J = 13.7, 3.9 Hz, 1H), 5.18 (m, 1H), 7.20 (d, J = 8.3 Hz, 2H), 7.27 (d, J = 8.8 Hz, 2H), 7.36 (d, J = 8.3 Hz, 2H), 7.82 (d, J = 8.3 Hz, 2H) and 7.89 (s, 1H); ¹³C (125 MHz, CDCl₃) δ 14.58, 20.76, 29.66, 31.17, 40.36, 41.16, 42.34, 53.33, 69.45, 75.85, 80.55, 121.14, 126.96, 128.67, 128.91, 129.41, 130.52, 132.65, 133.04, 133.58, 146.36 and 170.71. ESI' MS m/z 502 [M + H]⁺.

175: 1-(5-{1-[4-(4-methylphenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 2-(4-chlorophenyl)acetate: Azide 157 (0.0054 g, 0.0148 mmol)
was taken up in EtOH/H$_2$O (5:1, 0.200 mL). 4-ethynyl toluene 146 (0.0888 mL, 0.0052 g, 0.0444 mmol) was added as a solution in EtOH/H$_2$O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0045 mL, 0.0049 mmol) and 0.3 M aqueous solution of CuSO$_4$$\cdot$5H$_2$O (0.010 mL, 0.002 mmol) were added and the mixture was stirred at ambient temperature for 18 h. The next day, the mixture was purified directly by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 175 (0.0047 g, 66.2%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.89 (d, $J$ = 6.4 Hz, 3H), 1.24 (d, $J$ = 6.4 Hz, 3H), 1.44 (m, 1H), 1.56 (m, 1H), 1.56 (H$_2$O, br s), 1.68 (ddd, $J$ = 14.2, 8.3, 4.4 Hz, 1H), 1.79 (ddd, $J$ = 14.2, 8.8, 3.9 Hz, 1H), 1.85-1.97 (m, 2H), 1.96-2.06 (m, 1H), 2.38 (s, 3H), 3.37 (m, 1H), 3.57 (s, 2H), 3.70 (m, 1H), 4.34 (dd, $J$ = 13.7, 6.8 Hz, 1H), 4.54 (dd, 13.7, 3.9 Hz, 1H), 5.16 (m, 1H), 7.21 (dm, $J$ = 8.3 Hz, 4H), 7.27 (dm, $J$ = 8.3 Hz, 2H), 7.76 (dm, $J$ = 8.3 Hz, 2H) and 7.81 (s, 1H); $^{13}$C (125MHz, CDCl$_3$) $\delta$ 14.94, 20.73, 21.28, 29.55, 31.22, 40.34, 41.13, 42.36, 53.25, 69.58, 75.89, 80.63, 120.52, 125.61, 128.04, 128.65, 129.41, 130.57, 132.68, 132.99, 137.69, 147.50 and 170.64. ESI$^+$MS m/z 482 [M + H]$^+$. 

176: 1-(5-{1-[4-(3-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-

yl)propan-2-yl 2-(4-chlorophenyl)acetate: Azide 6p71 [H] (0.0055 g, 0.0150
mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL) and a 0.5 M solution of 3-ethynyl acetonilide 147 (0.0600 mL, 0.0048 g, 0.0300 mmol) in EtOH/H₂O (5:1) was added followed by a 1.1 M aqueous solution of ascorbate (0.0081 mL, 0.0016 g, 0.0090 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0150 mL, 0.0007 g, 0.0045 mmol) and the mixture was stirred at ambient temperature for 18 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL). Combined organic layers were washed with water (2 × 2 mL) and brine (2 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 176 (0.0033 g, 41.8%). ¹H NMR (500MHz, CDCl₃) δ 0.91 (d, J = 6.8 Hz, 3H), 1.25 (d, J = 6.4 Hz, 3H), 1.43 (m, 1H), 1.55 (m, 1H), 1.67 (ddd, J = 14.2, 8.8, 3.9 Hz, 1H), 1.80 (ddd, J = 14.2, 8.8, 3.4 Hz, 1H), 1.84-2.04 (m, 3H), 2.14 (s, 3H), 3.33 (m, 1H), 3.58 (s, 2H), 3.65 (m, 1H), 4.38 (dd, J = 13.7, 6.4 Hz, 1H), 4.51 (dd, J = 13.7, 3.9 Hz, 1H), 5.14 (m, 1H), 7.17 (dm, J = 8.8 Hz, 2H), 7.25 (dm, J = 8.8 Hz, 2H), 7.37 (t, J = 7.8 Hz, 1H), 7.50 (br s, 1H), 7.67 (dm, J = 7.8 Hz, 1H), 7.72-7.76 (m, 2H), 7.87 (s, 1H). ESI⁺MS m/z 525 [M + H]⁺.
177: 1-(5-{1-[4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 2-(4-chlorophenyl)acetate: Azide 157 (0.0054 g, 0.0148 mmol) was taken up in EtOH/H₂O (5:1, 0.200 mL). 4-ethynyl acetonilide 148 (0.087 mL, 0.0069 g, 0.0435 mmol) was added as a solution in EtOH/H₂O (5:1). Then, a 1.1 M aqueous solution of ascorbate (0.0045 mL, 0.0049 mmol) and 0.3 M aqueous solution of CuSO₄•5H₂O (0.010 mL, 0.002 mmol) were added and the mixture was stirred at ambient temperature for 18 h. The next day, the mixture was purified directly by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 177 (0.0017 g, 22.4%). ¹H NMR (500MHz, CDCl₃) δ 0.89 (d, J = 6.8 Hz, 3H), 1.24 (d, J = 6.8 Hz, 3H), 1.44 (m, 1H), 1.55 (H₂O, br s), 1.67 (ddd, J = 14.2, 8.8, 3.9 Hz, 1H), 1.79 (ddd, J = 14.2, 8.8, 4.4 Hz, 1H), 1.85-2.05 (m, 4H), 2.20 (s, 3H), 3.36 (m, 1H), 3.58 (s, 2H), 3.69 (m, 1H), 4.36 (dd, J = 13.7, 6.8 Hz, 1H), 4.54 (dd, J = 13.7, 3.9 Hz, 1H), 5.18 (m, 1H), 7.18 (br s, 1H), 7.20 (dm, J = 8.3 Hz, 2H), 7.28 (dm, J = 8.3 Hz, 2H), 7.53 (d, J = 8.3 Hz, 2H), 7.83 (d, J = 8.3 Hz, 2H) and 7.84 (s, 1H). ESI⁺MS m/z 525 [M + H]⁺.

Methyl (−)-nonactate (−)-3 (0.500 g, 2.312 mmol) was added to p-toluene sulfonylic acid (0.0044 g, 0.0231 mmol) as a solution in CH$_2$Cl$_2$ (10.0 mL). 3,4-Dihydro-2H-pyran (1.05 mL, 11.6 mmol) was added and the mixture was stirred at ambient temperature for 4 h. Water (10 mL) was added and the mixture was extracted with CH$_2$Cl$_2$ (3 × 5 mL). Combined organic layers were dried over MgSO$_4$ and concentrated to give a yellow oil, which was purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give 178 (0.8131 g, >99.9%). $^1$H NMR (500MHz, CDCl$_3$) δ 1.07-1.14 (m, 5H), 1.23 (d, J = 6.4 Hz, 2H), 1.44-1.75 (m, 13H), 1.81 (m, 1H), 1.90-2.05 (m, 2H), 2.51 (m, 1H), 3.47 (m, 1H), 3.68 (apparent d, J = 5.7 Hz, 3H), 3.84 (m, 1H), 3.89-4.07 (m, 3H), 4.68 (apparent dm, J = 55.3 Hz, 1H).


A suspension of lithium aluminum hydride (0.1963 g, 5.172 mmol) in THF (10.0 mL)
was cooled to 0 °C on an ice bath with stirring for 10 min. Ester 178 (0.8131 g, 2.586 mmol) was added dropwise as a solution in THF (3.0 mL) over the course of 10 min. The suspension was stirred at ambient temperature for 20 min. Slowly, water (0.400 mL) followed by 3.0 M aqueous NaOH (0.800 mL) and finally, more water (0.200 mL) were added as well as a couple extra milliliters of THF to facilitate stirring. This mixture was stirred for 5 min then filtered over Celite (4 g) in a sintered funnel. The Celite pad was rinsed with THF (2 × 5 mL). The resulting solution was concentrated to give a clear, sweet smelling oil, which was purified by flash chromatography and eluted in EtOAc/Hexanes (1:2) to give 179 (0.700 g, 99.4%) as a clear oil.

180: (2S)-2-[(2R,5S)-5-[(2S)-2-(oxan-2-yloxy)propyl]oxolan-2-yl]propyl 4-methylbenzene-1-sulfonate: A 3-necked flask was charged with 4-toluenesulfonyl chloride (0.980 g, 5.14 mmol). Alcohol 179 (0.700 g, 2.57 mmol) was added as a solution in pyridine (4.0 mL) and the solution was stirred at ambient temperature for 2.5 h, during which time a precipitate formed and the color of the solution changed from pale yellow to orange. Water (20 mL) and ether (2 mL) were added and the mixture was washed with saturated CuSO₄ (5 ×
3 mL). The aqueous layer was extracted with ether (3 mL). Combined organic layers were washed with saturated aqueous NH₄Cl (2 × 3 mL), water (2 × 3 mL) and brine (3 mL), dried over MgSO₄ then concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:3) to give 180 (0.594 g, 54.2%) as a clear oil that smelled vaguely of walnuts.

181: 2-[[((2S)-1-[(2S,5R)-5-[(2S)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-yl]oxy]oxane: Compound 180 (0.594 g, 1.39 mmol) and NaN₃ (0.1355 g, 2.085 mmol) were suspended in DMF (3.0 mL). This mixture was heated to 115 °C over 30 min, during which time the solvent turned yellow and the NaN₃ mostly dissolved. The mixture was then cooled to ambient temperature. Water (5 mL) was added and the mixture was extracted with ether (3 × 3 mL). Combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:9) to give 181 (0.3376 g, 81.7%) as a clear oil.
182: (2S)-1-[(2S,5R)-5-[(2S)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-ol: Azide 181 (0.3376 g, 1.14 mmol) was taken up in a 0.01 M solution of p-toluenesulfonic acid in MeOH (11.4 mL) and stirred at ambient temperature for 1 h. Saturated aqueous NaHCO₃ (5 mL) was added and the mixture was extracted with CH₂Cl₂ (3 × 5 mL). Combined organic layers were then washed with water (2 × 5 mL), dried over MgSO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give 182 (0.1627 g, 66.9%) as a clear oil.

183: (2S)-1-[(2S,5R)-5-[(2S)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: Activated zinc (0.2992 g, 4.577 mmol) was added to a solution of 1-(4-chlorophenyl)cyclopentane-1-carbonyl chloride 150 (0.3828 g, 1.526 mmol) in benzene (3 mL) and stirred rapidly enough to keep the zinc suspended at ambient temperature for 5 min. Then 182 (0.1627 g, 0.7628 mmol) was added as a solution in benzene (1.5 mL) and
reaction was stirred for 5 h. The reaction was not complete as determined by TLC (EtOAc/Hexanes 1:1), so more zinc (≈0.100 g, 0.0153 mmol) was added and the reaction stirred for an additional 18 h. Upon completion, the mixture was filtered over a cotton plug which was then rinsed with benzene. The solution was concentrated to give a crude residue which was purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give azidoester 183 (0.1954 g, 61.0%) as a white solid. IR (film-ATR): 3379, 2955, 2874, 1737, 1644, 1518, 1493, 1460, 1376, 1262, 1199, 1164, 1093, 1053, 1014 and 732 cm⁻¹.

\[
\begin{align*}
\text{OH} & \quad \text{O} \quad \text{OMe} \\
(+)\text{-3} & \quad \text{DHP} & \quad \text{CH}_2\text{Cl}_2 & \quad 184
\end{align*}
\]

184: methyl (2S)-2-[(2S,5R)-5-[(2R)-2-{oxan-2-yloxy}propyl]oxan-2-yl]propanoate:

Methyl (+)-nonactate (+)\text{-3} (0.150 g, 0.6935 mmol) was added to \textit{p}-toluene sulfonic acid (0.0013 g, 0.0069 mmol) as a solution in CH₂Cl₂ (3.0 mL). 3,4-Dihydro-2\textit{H}-pyran (0.317 mL, 3.468 mmol) was added and the mixture was stirred at ambient temperature for 4 h. Water (3 mL) was added and the mixture was extracted with CH₂Cl₂ (3 × 3 mL). Combined organic layers were dried over MgSO₄ and concentrated to give a yellow oil, which was purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give 184 (0.1778 g, 81.6%). ¹H NMR (500MHz, CDCl₃) δ 1.09-1.12 (m, 5H), 1.23 (d, J = 6.4 Hz, 2H),
1.46-1.75 (m, 12H), 1.80 (m, 1H), 1.91-2.05 (m, 2H), 2.51 (m, 1H), 3.47 (m, 1H),
3.68 (apparent d, J = 5.7 Hz, 3H), 3.84 (m, 1H), 3.89-4.07 (m, 3H), 4.68 (apparent
dm, J = 56.2 Hz, 1H).

185: (2R)-2-[(2S,5R)-5-[(2R)-2-(oxan-2-yloxy)propyl]oxolan-2-yl]propan-1-ol: A
suspension of lithium aluminum hydride (0.0429 g, 1.13 mmol) in THF (2.0 mL)
was cooled to 0 °C on an ice bath with stirring for 10 min. Ester 184 (0.1778 g,
0.565 mmol) was added dropwise as a solution in THF (1.0 mL) over the course
of 10 min. The suspension was stirred at ambient temperature for 20 min.
Slowly, water (0.043 mL) followed by 3.0 M aqueous NaOH (0.086 mL) and
finally, more water (0.043 mL) were added as well as a couple extra milliliters of
THF to facilitate stirring. This mixture was stirred for 5 min then filtered over
Celite (3 g) in a sintered funnel. The Celite pad was rinsed with THF (3 × 3 mL).
The resulting solution was concentrated to give a clear, sweet smelling oil, which
was purified by flash chromatography and eluted in EtOAc/Hexanes (1:2) to give
185 (0.353 g, >99.9%) as a clear oil.
186: (2R)-2-[(2S,5R)-5-[(2R)-2-(oxan-2-yloxy)propyl]oxolan-2-yl]propyl 4-methylbenzene-1-sulfonate: A 3-necked flask was charged with p-toluenesulfonyl chloride (0.3178 g, 1.95 mmol). Alcohol 185 (0.353 g, 1.30 mmol) was added as a solution in pyridine (2.0 mL). This solution was stirred at ambient temperature for 18 h, after which the reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1). More p-toluenesulfonyl chloride (0.1239 g, 0.650 mmol) was added and the mixture was stirred for an additional 24 h. Water (10 mL) and ether (3 mL) were added and the mixture was washed with saturated aqueous CuSO₄ (5 × 3 mL). The combined organic layers were extracted with ether (2 × 3 mL) and the combined organic layers were washed with saturated aqueous NH₄Cl (2 × 3 mL), water (2 × 3 mL) and brine (3 mL) then dried over MgSO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:3) to give 186 (0.1202 g, 21.7%) as a clear oil that smells vaguely of walnuts.
187: 2-[(2R)-1-[(2R,5S)-5-[(2R)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-yl]oxy]oxane: Compound 186 (0.1202 g, 0.2817 mmol) and NaN₃ (0.0275 g, 0.4225 mmol) were taken up in DMF (0.600 mL) and the suspension was heated to 100 °C for 45 min. The mixture was then cooled to ambient temperature, water (1 mL) was added and the mixture was extracted with ether (3 × 2 mL). Combined organic layers were dried over MgSO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:9) to give 187 (0.0524 g, 62.5%) as a clear oil.

188: (2R)-1-[(2R,5S)-5-[(2R)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-ol: Azide 187 (0.0524 g, 0.1761 mmol) was taken up in a 0.01 M solution of p-toluenesulfonic acid in MeOH (1.8 mL) and stirred at ambient temperature for 1 h. Saturated aqueous NaHCO₃ (2 mL) was added and the mixture was extracted with CH₂Cl₂ (3 × 2 mL). Combined organic layers were then washed with water (2 × 2 mL), dried over MgSO₄ and concentrated. The crude residue was purified by flash
chromatography and eluted in EtOAc/Hexanes (1:4) to give 188 (0.0241 g, 64.1%) as a clear oil.

189: (2R)-1-[(2R,5S)-5-[(2R)-1-azidopropan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: Azidoalcohol 188 (0.0241 g, 0.1129 mmol) was taken up in benzene (0.226 mL). A 0.5 M solution of 1-(4-chlorophenyl)cyclopentane-1-carbonyl chloride 150 (0.550 mL, 0.0549 g, 0.2258 mmol) in benzene was added followed by activated zinc (0.0443 g, 0.6774 mmol) was added and the suspension was stirred fast enough to keep the zinc suspended at ambient temperature for 18 h. The mixture was filtered over a cotton plug which was then rinsed with benzene (3 × 2 mL). The solution was then concentrated and the crude residue was purified by column chromatography and eluted in EtOAc/Hexanes (1:4) to give 189 (0.0221 g, 46.6%) as a white solid.
190: 

(2S)-1-[(2S,5R)-5-[(2S)-1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate:

Azidoester 183 (0.0114 g, 0.0271 mmol) was taken up in EtOH/H₂O (5:1, 0.250 mL). A 0.5 M solution of 2-ethynyl pyridine 143 (0.109 mL, 0.0056 g, 0.0542 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0493 mL, 0.0542 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0994 mL, 0.0048 g, 0.02981 mmol). This mixture was shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes 1:1), so more CuSO₄•5H₂O (0.010 mL, 0.033 mmol) was added and the mixture continued stirring for an additional 24 h. The reaction mixture was filtered through a plug of solid NaHCO₃ which was rinsed with EtOAc (4 × 2 mL) The yellow mixture was concentrated then purified by column chromatography and eluted in EtOAc/Hexanes (7:1) containing 1% Et₃N to give 190 (0.0069 g, 48.6%). [α]₂⁰<sup>D</sup> = +41.74°; 1H NMR (500 MHz, CDCl₃) δ 0.84 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.30 (m, 1H), 1.49 (m, 1H), 1.55-1.93 (m, 10H), 1.98 (m, 1H), 2.60 (m, 1H), 2.71 (m, 1H), 3.35 (m, 1H), 3.42 (m, 1H), 4.30 (dd, J = 13.9, 8.1 Hz, 1H), 4.60 (dd, J = 13.2, 4.4 Hz, 1H), 5.00 (m, 1H), 7.22 (m, 1H), 7.25 (dm, J = 8.8 Hz, 2H), 7.33 (dm, J = 8.8 Hz, 2H), 7.77 (dt, J = 8.1, 1.5 Hz,
1H), 8.15 (s, 1H), 8.18 (dm, J = 8.1 Hz, 1H) and 8.59 (m, 1H). IR (film-ATR): 2961, 2875, 1719, 1604, 1492, 1472, 1421, 1233, 1184, 1167, 1121, 1092, 1062, 1038, 1014, 785 and 534 cm\(^{-1}\). ESI\(^+\)MS m/z 523 [M + H]\(^+\).

![Chemical Structure](image)

191: \((2S)-1-[(2S,5R)-5-[(2S)-1-[4-(3-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl\) 1-(4-chlorophenyl)cyclopentane-1-carboxylate:

Azidoester 183 (0.0113 g, 0.0269 mmol) was taken up in EtOH/H\(_2\)O (5:1, 0.250 mL). Then, a 0.5 M solution of 3-ethynyl acetanilide 147 (0.1076 mL, 0.0086 g, 0.0538 mmol) in EtOH/H\(_2\)O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0489 mL, 0.0095 g, 0.0538 mmol) and a 0.3 M aqueous solution of CuSO\(_4\)•5H\(_2\)O (0.0986 mL, 0.0047 g, 0.0296 mmol). This mixture was then shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1), so more CuSO\(_4\)•5H\(_2\)O (0.010 mL, 0.033 mmol) was added and the mixture was stirred for an additional 24 h. The mixture was then filtered over a plug of solid NaHCO\(_3\) which was then rinsed with EtOAc (4 × 2 mL). The yellow mixture was concentrated then purified by column chromatography and eluted in EtOAc/Hexanes (2:1) to give triazoloester 191 (0.0107 g, 68.6%). \([\alpha]_{D}^{25} = +28.33^\circ\); \(^{1}H\) NMR (500MHz, CDCl\(_3\)) \(\delta\) 0.89 (d, J =
7.3 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.29 (m, 1H), 1.48 (m, 1H), 1.57 (ddd, J = 13.9, 9.5, 3.7 Hz, 1H), 1.64-1.98 (m, 9H), 2.19 (s, 3H), 2.57 (m, 1H), 2.67 (m, 1H), 3.18 (m, 1H), 3.27 (m, 1H), 4.35 (dd, J = 13.9, 6.6 Hz, 1H), 4.48 (dd, J = 13.9, 4.4 Hz, 1H), 5.03 (m, 1H), 7.19 (dm, J = 8.8 Hz, 2H), 7.24 (dm, J = 8.8 Hz, 2H), 7.39 (t, J = 8.1 Hz, 1H), 7.59 (br s, 1H), 7.69 (dm, J = 7.3 Hz, 1H), 7.76 (m, 1H), 7.78 (dm, J = 7.3 Hz, 1H) and 7.85 (s, 1H). IR (film-ATR): 3300, 3274, 3128, 2966, 2875, 1717, 1673, 1618, 1592, 1569, 1535, 1490, 1450, 1403, 1371, 1309, 1253, 1185, 1167, 1121, 1093, 1044, 1014, 967, 831, 790, 754, 719, 693, 666, 609 and 537 cm⁻¹. ESI’MS m/z 579 [M + H]⁺.

192: (2S)-1-[(2S,5R)-5-[(2S)-1-[4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl 1-[(4-chlorophenyl)cyclopentane-1-carboxylate:

Azidoester 183 (0.0113 g, 0.0269 mmol) was taken up in EtOH/H₂O (5:1, 0.250 mL). Then, a 0.5 M solution of 4-ethynyl acetanilide 148 (0.1076 mL, 0.0086 g, 0.0538 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0489 mL, 0.0095 g, 0.0538 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0986 mL, 0.0047 g, 0.0296 mmol). This mixture was
then shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1), so more CuSO₄•5H₂O (0.010 mL, 0.033 mmol) was added and the mixture was stirred for an additional 24 h. The mixture was then filtered over a plug of solid NaHCO₃ which was then rinsed with EtOAc (4 × 2 mL). The yellow mixture was concentrated then purified by column chromatography and eluted in EtOAc/Hexanes (2:1) to give triazoloester 192 (0.0025 g, 16.0%). [α]²³ = + 22.89°; ¹H NMR (500MHz, CDCl₃) δ 0.87 (d, J = 6.6 Hz, 3H), 1.13 (d, J = 6.6 Hz, 3H), 1.30 (m, 1H), 1.49 (m, 1H), 1.56 (H₂O, br s), 1.57 (m, 1H), 1.62-1.70 (m, 1H), 1.70-1.98 (m, 8H), 2.20 (s, 3H), 2.61 (m, 1H), 2.70 (m, 1H), 3.24 (m, 1H), 3.33 (m, 1H), 4.35 (dd, J = 13.9, 6.6 Hz, 1H), 4.50 (dd, J = 13.9, 3.7 Hz, 1H), 5.09 (m, 1H), 7.20 (br s, 1H), 7.22 (dm, J = 8.8 Hz, 2H), 7.27 (dm, J = 8.8 Hz, 2H), 7.58 (d, J = 8.1 Hz, 2H), 7.86 (s, 1H) and 7.88 (d, J = 8.1 Hz, 2H). IR (film-ATR): 3279, 3262, 3111, 2957, 2925, 2858, 1716, 1673, 1599, 1562, 1534, 1497, 1455, 1411, 1370, 1316, 1253, 1182, 1121, 1094, 1065, 1014, 972, 833, 761, 576 and 551 cm⁻¹. ESI⁺MS m/z 579 [M + H]⁺.

\[\text{192} \quad \text{(2R)-1-[(2R,5S)-5-[(2R)-1-[4-(pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl}} \quad \text{1-(4-chlorophenyl)cyclopentane-1-carboxylate:} \]
Azidoester 188 (0.0074 g, 0.0176 mmol) was taken up in EtOH/H2O (5:1, 0.250 mL). A 0.5 M solution of 2-ethynyl pyridine 143 (0.0704 mL, 0.0036 g, 0.0352 mmol) in EtOH/H2O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.032 mL, 0.0062 g, 0.0352 mmol) and a 0.3 M aqueous solution of CuSO4•5H2O (0.0647 mL, 0.0031 g, 0.0194 mmol). This mixture was shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1), so more CuSO4•5H2O (0.010 mL, 0.003 mmol) was added and the mixture was stirred for a further 24 h. The mixture was then filtered through a plug of solid NaHCO3 which was then rinsed with EtOAc (4 × 2 mL). The mixture was then concentrated and purified by column chromatography and eluted in EtOAc/Hexanes (7:1) containing 1% v/v Et3N to give 193 (0.0039 g, 42.4%). \( [\alpha]_D^23 = -17.69^\circ \); \(^1\)H NMR (500MHz, CDCl3) \( \delta \) 0.84 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.26 (grease, br s), 1.49 (m, 1H), 1.59 (H2O, br s), 1.60-1.66 (m, 1H), 1.66-1.93 (m, 9H), 1.98 (m, 1H), 2.59 (m, 1H), 2.71 (m, 1H), 3.34 (m, 1H), 3.42 (m, 1H), 4.30 (dd, J = 13.9, 7.3 Hz, 1H), 4.60 (dd, J = 13.9, 4.4 Hz, 1H), 5.00 (m, 1H), 7.22 (m, 1H), 7.25 (dm, J = 8.8 Hz, 2H), 7.33 (dm, J = 8.8 Hz, 2H), 7.77 (dt, J = 8.1, 1.5 Hz, 1H), 8.15 (s, 1H), 8.18 (d, J = 8.1 Hz, 1H) and 8.59 (dm, J = 5.1 Hz, 1H). IR (film-ATR): 2965, 2874, 1719, 1603, 1492, 1459, 1420, 1381, 1234, 1185, 1120, 1092, 1039, 1014, 975, 833, 786, 718 and 535 cm\(^{-1}\). ESI\(^+\)MS \( m/z \) 523 [M + H]\(^+\).
194: (2R)-1-[(2R,5S)-5-[(2R)-1-4-(3-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate:

Azidoester 188 (0.0066 g, 0.0157 mmol) was taken up in EtOH/H2O (5:1, 0.250 ml). A 0.5 M solution of 3-ethylthyl acetanilide 147 (0.0628 mL, 0.005 g, 0.0314 mmol) in EtOH/H2O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0285 mL, 0.0055 g, 0.0314 mmol) and a 0.3 M aqueous solution of CuSO4•5H2O (0.0577 mL, 0.0028 g, 0.0173 mmol). This mixture was shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1), so more CuSO4•5H2O (0.010 mL, 0.003 mmol) was added and the mixture was stirred for a further 24 h. The mixture was then filtered through a plug of solid NaHCO3 which was then rinsed with EtOAc (4 × 2 mL). The mixture was then concentrated and purified by column chromatography and eluted in EtOAc/Hexanes (2:1) to give 194 (0.0049 g, 53.8%). [α]D23 = −20.63°;

1H NMR (500MHz, CDCl3) δ 0.90 (d, J = 6.6 Hz, 3H), 1.14 (d, J = 6.6 Hz, 3H), 1.25 (grease, br s), 1.29 (m, 1H), 1.48 (m, 1H), 1.56 (m, 1H), 1.60 (H2O, br s), 1.63-1.82 (m, 7H), 1.81-1.97 (m, 3H), 2.19 (s, 3H), 2.58 (m, 1H), 2.67 (m, 1H), 3.18 (m, 1H), 3.26 (m, 1H), 4.36 (dd, J = 13.9, 6.6 Hz, 1H), 4.48 (dd, J = 13.9, 4.4 Hz, 1H), 5.02 (m, 1H), 7.19 (dm, J = 8.8 Hz, 2H), 7.24 (dm, J = 8.8 Hz, 2H), 7.40 (t, J = 8.1 Hz,
1H), 7.54 (br s, 1H), 7.69 (dm, J = 8.1 Hz, 1H), 7.76 (m, 1H), 7.79 (dm, J = 8.1 Hz, 1H) and 7.85 (s, 1H). IR (film-ATR): 3309, 3131, 2953, 1717, 1696, 1570, 1536, 1490, 1234, 1167 and 1094 cm⁻¹. ESI^+MS m/z 579 [M + H]^+.

195: (2R)-1-[(2R,5S)-5-[(2R)-1-[4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate:
Azidoester 188 (0.0066 g, 0.0157 mmol) was taken up in EtOH/H₂O (5:1, 0.250 mL). A 0.5 M solution of 4-ethynyl acetonilide 148 (0.0628 mL, 0.005 g, 0.0314 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0285 mL, 0.0055 g, 0.0314 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.0577 mL, 0.0028 g, 0.0173 mmol). This mixture was shaken at 225 RPM at 37 °C for 18 h. Reaction was still incomplete as determined by TLC (EtOAc/Hexanes, 1:1), so more CuSO₄•5H₂O (0.010 mL, 0.003 mmol) was added and the mixture was stirred for a further 24 h. The mixture was then filtered through a plug of solid NaHCO₃ which was then rinsed with EtOAc (4 × 2 mL). The mixture was then concentrated and purified by column chromatography and eluted in EtOAc/Hexanes (2:1) to give 195 (0.0014 g, 15.6%). [α]D^23 = + 12.77°; ^1H NMR (500MHz, CDCl₃) δ 0.87 (d, J = 6.6 Hz, 3H), 1.13 (d, J = 6.6 Hz, 3H), 1.25
(grease, br s), 1.28 (m, 1H), 1.45-1.60 (m, 2H), 1.55 (H₂O, br s), 1.62-1.99 (m, 10H), 2.20 (s, 3H), 2.61 (m, 1H), 2.70 (m, 1H), 3.24 (m, 1H), 3.33 (m, 1H), 4.35 (dd, J = 13.9, 6.6 Hz, 1H), 4.51 (dd, J = 13.9, 3.7 Hz, 1H), 5.09 (m, 1H), 7.18 (br, s, 1H), 7.22 (dm, J = 8.8 Hz, 2H), 7.28 (dm, J = 8.8 Hz, 2H), 7.58 (dm, J = 8.8 Hz, 2H), 7.86 (s, 1H) and 7.88 (dm, J = 8.8 Hz, 2H). IR (film-ATR): 3280, 3116, 3052, 2927, 2854, 1717, 1673, 1599, 1562, 1533, 1497, 1455, 1410, 1370, 1315, 1253, 1184, 1122, 1093, 1063, 1014, 973, 833, 760, 719, 604, 576 and 552 cm⁻¹. ESI⁺MS m/z 579 [M + H]⁺.

\[
\text{OH} \quad \text{O} \quad \text{OMe} \quad \text{PPh₃-Br₂} \quad \text{CH₂Cl₂} \quad \text{Br} \quad \text{O} \quad \text{OMe}
\]

196: methyl 2-[5-(2-bromopropyl)oxolan-2-yl]propanoate: Triphenylphosphine (1.1606 g, 4.425 mmol) was taken up in CH₂Cl₂ (8 mL) and the solution was cooled to 0 °C on an ice bath. Bromine (0.2267 mL, 4.425 mmol) was added dropwise, the solution turned pale yellow and a precipitate formed. This suspension was stirred at 0 °C for 30 min, then 3 (0.637 g, 2.95 mmol) was added as a solution in CH₂Cl₂ (2 mL). The mixture was stirred at 0 °C for an additional 20 min and then was warmed to ambient temperature and stirred for 4 h, during which time the reaction became a dark amber color. The mixture was then filtered over a silica plug and eluted with ether. The filtrate was then purified by flash chromatography and eluted in EtOAc/Hexanes (1:1) to give
196 (0.5497 g, 66.7%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 1.10 (d, J = 7.3 Hz, 3H), 1.48 (m, 1H), 1.62 (m, 1H), 1.71 (d, J = 6.6 Hz, 3H), 1.86 (m, 1H), 1.93-2.07 (m, 2H), 2.21 (m, 1H), 2.50 (m, 1H), 3.69 (s, 3H), 4.01 (m, 1H) and 4.18 (hex, J = 6.6 Hz, 1H); $^{13}$C (125MHz, CDCl$_3$) $\delta$ 13.45, 25.96, 28.35, 30.71, 45.34, 47.18, 47.48, 51.64, 77.22, 80.58 and 175.23. IR (film-ATR): 2976, 2950, 2881, 1739, 1460, 1435, 1378, 1260, 1199, 1164 and 1081 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{11}$H$_{20}$O$_3$Br: 279.0596; found 279.0590 ([M + H]$^+$, Δppm 2.1).

![Chemical structure of 196 and 197]

**197: 2-[5-(2-bromopropyl)oxolan-2-yl]propan-1-ol:** Lithium aluminum hydride (0.1496 g, 3.94 mmol) was taken up in THF (5 mL) and cooled to 0 °C on an ice bath for 5 min. Ester 196 (0.5497 g, 1.97 mmol) was slowly added as a solution in THF (5 mL). The suspension was removed from the ice bath and stirred at ambient temperature for 15 min, after which time TLC (EtOAc/Hexanes, 1:1) showed consumption of starting material. The reaction was quenched by the addition of water (0.150 mL), 3.0 M aqueous NaOH (0.300 mL) and water (0.150 mL). This suspension was stirred for 10 min, then filtered over Celite (5 g) which was rinsed with THF. The filtrate was concentrated and the crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:2) to give 197 (0.4431 g, 89.6%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.81 (d, J = 7.3 Hz, 3H), 1.48 (m,
1H), 1.59 (m, 1H), 1.73 (d, J = 6.6 Hz, 3H), 1.90 (m, 1H), 1.94-2.06 (m, 2H), 2.21 (m, 1H), 3.28 (dd, J = 8.1, 3.7 Hz, 1H), 3.53-3.62 (m, 2H), 3.64 (m, 1H), 3.94 (m, 1H), 4.03 (m, 1H), 4.13 (m, 1H). ESI*MS m/z 251 [M + H]⁺; 253 [M + 2]⁺. HRMS (ESI) m/z calcd. for C_{10}H_{20}O_{2}Br: 251.0647; found 251.0649 ([M + H]⁺, Δppm 0.9).

198: 2-[5-(2-azidopropyl)oxolan-2-yl]propan-1-ol: Alcohol 197 (0.4431 g, 1.76 mmol) was taken up in DMF (13 mL) and the solution was heated to 60 °C. Sodium azide (0.175 g, 2.64 mmol) was added and the mixture was stirred for 2 h, then cooled to ambient temperature and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:2) to give 198 (0.2604 g, 69.4%). 

\[ \ \text{Br} \quad \begin{array}{c} \text{NaN}_3 \\
\text{DMF} \end{array} \quad \begin{array}{c} \text{OH} \\
\text{O} \end{array} \quad \begin{array}{c} \text{N}_3 \\
\text{OH} \end{array} \quad 197 \quad \begin{array}{c} \text{198} \\
\text{Br} \quad \text{OH} \quad \text{OH} \end{array} \]

1H NMR (600MHz, CDCl₃) δ 0.88 (d, J = 7.0 Hz, 3H), 1.27 (d, 7.0 Hz, 3H), 1.48 (m, 1H), 1.58 (m, 1H), 1.61 (contaminant? t J = 6.5 Hz, 2H), 1.71 (m, 1H), 1.83 (m, 1H), 1.93-2.04 (m, 2H), 3.29 (dd, J = 8.8, 2.3 Hz, 1H), 3.53-3.68 (m, 4H) and 3.96 (m, 1H); 

\[ {^{13}}\text{C} \quad \text{150MHz, CDCl₃} \quad \text{δ} \quad 13.58, 20.06, 30.26, 40.94, 42.73, 55.58, 68.31, 76.56 \text{ and } 85.48. \]

IR (film-ATR): 3435, 2967, 2880, 2102, 1248 and 1038 cm⁻¹. ESI*MS m/z 214 [M + H]⁺.
199: **2-[5-(2-aminopropyl)oxolan-2-yl]propan-1-ol:** A solution of 198 (0.2428 g, 1.14 mmol) in MeOH (3 mL) was added to a stirred suspension of 10 % Pd/C (0.024 g) in MeOH (7 mL) under an atmosphere of hydrogen. This mixture was stirred at ambient temperature for 18 h. The reaction mixture was then filtered over Celite (4 g) through a sintered funnel. The Celite pad was rinsed with MeOH (4 × 5 mL) and the filtrate was concentrated. The crude residue was purified by filtering over a silica plug that was rinsed EtOAc/Hexanes (1:1) then eluted with MeOH/CH₂Cl₂ (1:19). The purified filtrate was concentrated to give the 199 (0.1906 g, 89.2%). ¹H NMR (600MHz, CDCl₃) δ 0.81 (d, J = 7.0 Hz, 3H), 1.08 (d, J = 6.5 Hz, 3H), 1.43-1.53 (m, 2H), 1.55-1.67 (m, 3H), 1.71 (m, 1H), 1.91-2.03 (m, 3H), 2.41 (br s, 2H), 3.06 (m, 1H), 3.54-3.61 (m, 2H), 3.63 (m, 1H) and 3.99 (m, 1H); ¹³C (150MHz, CDCl₃) δ 13.70, 23.64, 30.35, 30.81, 40.96, 44.41, 45.70, 68.33, 77.12 and 85.53. IR (film-ATR): 3354, 2961, 2877, 1596, 1459, 1377, 1043 and 751.

200: **1-(4-chlorophenyl)-N-[1-[5-(1-hydroxypropan-2-yl)oxolan-2-yl]propan-2-yl]cyclopentane-1-carboxamide:** Aminoalcohol 199 (0.1906 g, 1.02 mmol) was
taken up in CH₂Cl₂ (8 mL) and Et₃N (0.143 mL, 1.02 mmol) was added. This mixture was cooled to 0 °C and stirred for 15 min. 1-(4-chlorophenyl)cyclopentane-1-carbonyl chloride 150 (0.293 g, 1.21 mmol) was added as a solution in CH₂Cl₂ (2 mL) over the course of 1 h with the temperature being maintained at 0 °C. The reaction was warmed to room temperature and stirred for an additional 1.5 h. Water (10 mL) was added and the mixture was extracted with CH₂Cl₂ (4 × 5 mL). Combined organic layers were dried over Na₂SO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:1) to give 200 (0.2224 g, 55.4%). ¹H NMR (500MHz, CDCl₃) δ 0.80 (d, J = 6.8 Hz, 3H), 1.04 (d, J = 6.8 Hz, 3H), 1.37 (m, 1H), 1.47-1.58 (m, 3H), 1.58-1.71 (m, 3H), 1.73-1.81 (m, 2H), 1.83 (m, 1H), 1.87-1.97 (m, 3H), 2.37-2.49 (m, 2H), 2.96 (br s, 1H), 3.51-3.61 (m, 3H), 3.75 (m, 1H), 4.02 (m, 1H), 5.41 (d, J = 7.8 Hz, 1H) and 7.26-7.31 (m, 4H); ¹³C NMR (125MHz, CDCl₃) δ 13.48, 20.25, 23.80, 29.70, 30.80, 36.63, 36.67, 40.69, 41.86, 43.78, 58.90, 67.94, 76.84, 84.79, 128.11, 128.61, 132.49, 142.97 and 174.76. IR (film-ATR): 3471, 3310, 3056, 2960, 2875, 1638, 1534, 1489, 1457, 1402, 1350, 1270, 1170, 1136, 1089, 1041 cm⁻¹. ESI⁺MS: m/z 394 [M + H]⁺. HRMS (ESI) m/z calcld. for C₂₂H₃₃NO₃Cl: 394.2149; found 394.2185 ([M + H]⁺, Δppm 9.1).
201: 2-[5-(2-[(1-(4-chlorophenyl)cyclopentyl]formamido)propyl]oxolan-2-yl]propyl

1-(4-chlorophenyl)cyclopentane-1-carboxylate: Another spot appeared that stained a similar blue color in anisaldehyde that was UV active on the TLC plate around Rf = 0.6. This spot was collected as well and determined to be the bis-addition, amidoester product, 201 (0.0537 g) $^1$H NMR (500MHz, CDCl$_3$) δ 0.71 (d, J = 6.8 Hz, 3H), 1.02 (d, J = 6.8 Hz, 3H), 1.28 (m, 1H), 1.39 (m, 1H), 1.46 (m, 1H), 1.55-1.66 (m, 3H), 1.66-1.78 (m, 8H), 1.78-1.93 (m, 5H), 2.32-2.42 (m, 2H), 2.62-2.69 (m, 2H), 3.42 (q, J = 7.3 Hz, 1H), 3.77 (m, 1H), 3.84 (dd, J = 10.8, 7.3 Hz, 1H), 4.01 (dd, J = 10.8, 4.9 Hz, 1H), 4.04 (m, 1H), 6.03 (d, J = 8.3 Hz, 1H), 7.18-7.23 (m, 4H), 7.23-7.26 (d, J = 8.8 Hz, 2H) and 7.29-7.32 (d, J = 8.8 Hz, 2H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 12.77, 19.02, 23.45, 23.48, 23.67, 27.66, 31.58, 35.88, 36.00, 36.46, 36.51, 37.42, 40.46, 43.85, 58.73, 58.79, 67.38, 75.93, 80.61, 127.94, 128.24, 128.32, 128.41, 132.22, 132.53, 141.83, 143.07, 174.45 and 175.32. IR (film-ATR): 3399, 2960, 2875, 1724, 1651, 1495, 1455, 1396, 1237, 1165, 1094, 1043, 1012, 964, 911, 827 and 754 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{34}$H$_{44}$NO$_4$Cl$_2$: 600.2647; found 600.2621 ([M + H]$^+$, Δppm 4.4).
202: 2-[5-[(2-[[1-(4-chlorophenyl)cyclopentyl]formamido]propyl]oxolan-2-yl]propyl 4-methylbenzene-1-sulfonate: Amidoalcohol 200 (0.2224 g, 0.5645 mmol) was dissolved in pyridine (1 mL). p-Toluenesulfonfyl chloride (0.2152 g, 1.129 mmol) was added and the mixture was stirred at ambient temperature for 5 h. Water (3 mL) and ether (3 mL) were added and the mixture was washed with saturated aqueous CuSO$_4$•5H$_2$O (6 x 3 mL), saturated aqueous NaHCO$_3$ (3 x 3 mL) and water (2 x 3 mL). Combined organic layers were dried over MgSO$_4$ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:2) to give 202 (0.2118 g, 68.5%) as a clear oil. $^1$H NMR (500MHz, CDCl$_3$) δ 0.83 (d, J = 6.8 Hz, 3H), 1.01 (d, J = 6.8 Hz, 3H), 1.30 (m, 1H), 1.38-1.47 (m, 2H), 1.55 (ddd, J = 14.2, 5.4, 3.9 Hz, 1H), 1.60-1.69 (m, 2H), 1.69-1.79 (m, 4H), 1.80-1.93 (m, 3H), 2.31-2.42 (m, 2H), 2.44 (s, 3H), 3.54 (q, J = 7.3 Hz, 1H), 3.74 (m, 1H), 3.85 (dd, J = 9.3, 6.4 Hz, 1H), 3.99 (m, 1H), 4.04 (dd, J = 9.3, 4.9 Hz, 1H), 5.78 (d, J = 8.3 Hz, 1H), 7.22-7.26 (m, 4H), 7.34 (d, J = 8.3 Hz, 2H) and 7.78 (dm, J = 8.3 Hz, 2H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 12.84, 19.46, 21.64, 23.77, 28.04, 31.49, 36.61, 36.67, 38.02, 40.92, 43.91, 58.86, 72.73, 76.23, 77.22, 79.92, 127.87, 128.04, 128.53, 129.81, 132.35, 133.01, 143.07, 144.70 and 174.62. IR (film-ATR): 3403, 2965, 2876, 1650, 1598, 1510, 1455, 1358, 1176, 1095, 1017, 962, 820, 753, 666 and 554 cm$^{-1}$. ESI$^+$MS m/z 548 [M + H]$^+$. 

199
203: N-{1-[5-(1-azidopropan-2-yl)oxolan-2-yl]propan-2-yl}-1-(4-chlorophenyl)cyclopentane-1-carboxamide: Amide 202 (0.2030 g, 0.3703 mmol) was taken up in DMF (1 mL) and heated to 95 °C for 30 min. The mixture was then cooled to ambient temperature. Water (2 mL) was added and the mixture was extracted with EtOAc (3 × 3 mL), dried over MgSO₄ and concentrated. The crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:4) to give 203 (0.1288 g, 83.0%) as a white solid. $^1$H NMR (500MHz, CDCl₃) δ 0.90 (d, J = 6.8 Hz, 3H), 1.06 (d, J = 6.8 Hz, 3H), 1.36 (m, 1H), 1.43-1.55 (m, 2H), 1.61 (m, 1H), 1.64-1.72 (m, 3H), 1.73-1.82 (m, 2H), 1.81-1.95 (m, 4H), 2.37-2.46 (m, 2H), 3.13 (dd, J = 11.7, 7.3 Hz, 1H), 3.34 (dd, J = 12.2, 4.4 Hz, 1H), 3.56 (q, J = 7.3 Hz, 1H), 3.82 (m, 1H), 4.06 (m, 1H), 5.88 (d, J = 7.8 Hz, 1H) and 7.24-7.30 (m, 4H); $^{13}$C NMR (125MHz, CDCl₃) δ 14.04, 19.38, 23.81, 28.25, 31.55, 36.72, 38.78, 40.86, 43.93, 55.02, 58.89, 76.15, 81.09, 127.98, 128.54, 132.38, 143.11 and 174.60. IR (film-ATR): 3342, 2963, 2875, 2096, 1644, 1498, 1453, 1276 and 1095 cm⁻¹. ESI'MS m/z 419 [M + H]⁺.
204: 1-(4-chlorophenyl)-N-[1-(5-[1-(4-pyridin-2-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl]cyclopentane-1-carboxamide: Azide 203 (0.017 g, 0.0406 mmol) was taken up in EtOH/H$_2$O (5:1, 0.500 mL). A 0.5 M solution of 2-ethynyl pyridine 143 (0.191 mL, 0.0098 g, 0.0954 mmol) in EtOH/H$_2$O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0086 mL, 0.0017 g, 0.0095 mmol) and a 0.3 M aqueous solution of CuSO$_4$•5H$_2$O (0.016 mL, 0.0008 g, 0.0048 mmol). This mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0086 mL, 0.0095 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO$_3$ which was then rinsed with EtOAc (5 × 2 mL). The filtrate was concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (2:1) to give 204 (0.0173 g, 64.5%). $^1$H NMR (500MHz, CDCl$_3$) δ 0.84 (d, J = 6.8 Hz, 3H), 1.07 (d, J = 6.8 Hz, 3H), 1.38 (m, 1H), 1.48-1.57 (m, 2H), 1.60-1.72 (m, 3H), 1.72-1.82 (m, 2H), 1.84-1.95 (m, 4H), 2.04 (m, 1H), 2.39-2.49 (m, 2H), 3.49 (q, J = 7.3 Hz, 1H), 3.77 (m, 1H), 4.04 (m, 1H), 4.24 (dd, J = 13.7, 7.8 Hz, 1H), 4.49 (dd, J = 13.7, 4.9 Hz, 1H), 5.67 (d, J = 7.8 Hz, 1H), 7.21-7.29 (m, 4H), 7.77 (dt, J = 7.8, 1.5 Hz, 1H), 8.13 (s, 1H), 8.18 (d, J = 7.8 Hz, 1H) and 8.57 (dm, J = 201
4.9 Hz, 1H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 13.95, 19.90, 23.81, 23.83, 28.66, 31.38, 36.70, 36.75, 39.72, 41.36, 43.92, 53.60, 58.93, 76.46, 77.20, 80.91, 120.21, 122.76, 122.80, 128.03, 128.56, 132.41, 136.87, 143.08, 148.18, 149.34, 150.35 and 174.77. IR (film-ATR): 3359, 3056, 2962, 2874, 1648, 1601, 1518, 1459, 1373, 1231, 1092, 1042, 785 and 752 cm$^{-1}$. HRMS (ESI) $m/z$ calcd. for C$_{29}$H$_{37}$N$_5$O$_2$Cl: 522.2636; found 522.2620 ([M + H]$^+$, Δppm 3.0).

205: 1-(4-chlorophenyl)-N-[1-(5-[1-[4-(3-acetamidophenyl)-1H-1,2,3-triazol-1-yl]propan-2-yl]oxolan-2-yl]propan-2-yl]cyclopentane-1-carboxamide: Azide 203 (0.017 g, 0.0406 mmol) was taken up in EtOH/H$_2$O (5:1, 0.500 mL). A 0.5 M solution of 3-ethynyl acetonilide 147 (0.191 mL, 0.0152 g, 0.0954 mmol) in EtOH/H$_2$O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0086 mL, 0.0017 g, 0.0095 mmol) and a 0.3 M aqueous solution of CuSO$_4$•5H$_2$O (0.016 mL, 0.0008 g, 0.0048 mmol). This mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0086 mL, 0.0095 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO$_3$ which was then rinsed with EtOAc (5 × 2 mL). The
filtrate was concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (2:1) to give 205 (0.0095 g, 34.4%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.88 (d, J = 6.8 Hz, 3H), 1.05 (d, J = 6.4 Hz, 3H), 1.25 (grease, br s), 1.35 (m, 1H), 1.43-1.57 (m, 3H), 1.57-1.70 (m, 3H), 1.70-1.87 (m, 3H), 1.87-2.03 (m, 4H), 2.19 (s, 3H), 2.37-2.49 (m, 2H), 3.39 (m, 1H), 3.63 (m, 1H), 4.10 (m, 1H), 4.30 (dd, J = 13.7, 6.8 Hz, 1H), 4.42 (dd, J = 13.7, 4.9 Hz, 1H), 5.47 (d, J = 8.3 Hz, 1H), 7.20-7.27 (m, 4H), 7.38 (t, J = 7.8 Hz, 1H), 7.67-7.74 (m, 2H), 7.84 (m, 1H) and 7.87 (s, 1H); $^{13}$C NMR (125MHz, CDCl$_3$) $\delta$ 14.46, 20.49, 23.78, 24.66, 24.67, 29.11, 31.36, 36.61, 36.70, 36.75, 39.87, 41.79, 43.90, 53.42, 59.01, 76.35, 80.96, 116.67, 119.36, 121.27, 121.36, 128.00, 128.63, 129.58, 131.39, 132.54, 138.55, 142.89, 146.98, 168.45 and 175.09. IR (film-ATR): 3305, 2962, 2873, 1643, 1569, 1528, 1490, 1450, 1372, 1313, 1271, 1226, 1093, 1044, 791 and 753 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{32}$H$_{41}$N$_5$O$_3$Cl: 578.2898; found 578.2888 ([M + H]$^+$, Δppm 1.7).

![Chemical Reaction Diagram]

206: 1-(4-chlorophenyl)-N-[1-{5-[1-{4-(4-acetamidophenyl)-1H-1,2,3-triazol-1-yl}propan-2-yl]oxolan-2-yl}propan-2-yl]cyclopentane-1-carboxamide: Azide

203 (0.017 g, 0.0406 mmol) was taken up in EtOH/H$_2$O (5:1, 0.500 mL). A 0.5 M solution of 4-ethynyl acetonilide 148 (0.191 mL, 0.0152 g, 0.0954 mmol) in 203
EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0086 mL, 0.0017 g, 0.0095 mmol) and a 0.3 M aqueous solution of CuSO₄•5H₂O (0.016 mL, 0.0008 g, 0.0048 mmol). This mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0086 mL, 0.0095 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO₃ which was then rinsed with EtOAc (5 × 2 mL). The filtrate was concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (2:1) to give 206 (0.0156 g, 56.5%). ¹H NMR (500MHz, CDCl₃) δ 0.85 (d, J = 6.8 Hz, 3H), 1.05 (d, J = 6.8 Hz, 3H), 1.37 (m, 1H), 1.47-1.57 (m, 3H), 1.61-1.72 (m, 2H), 1.74-1.82 (m, 2H), 1.82-1.96 (m, 4H), 2.00 (m, 1H), 2.19 (s, 3H), 2.38-2.50 (m, 2H), 3.42 (m, 1H), 3.70 (m, 1H), 4.14 (m, 1H), 4.28 (dd, J = 13.7, 7.3 Hz, 1H), 4.44 (dd, J = 13.7, 4.4 Hz, 1H), 5.50 (d, J = 8.8 Hz, 1H), 7.20-7.27 (m, 4H), 7.55-7.60 (m, 3H) and 7.82-7.88 (m, 3H); ¹³C NMR (125MHz, CDCl₃) δ 14.10, 20.34, 23.83, 24.65, 28.89, 31.34, 36.75, 36.82, 39.76, 41.87, 43.67, 53.29, 58.98, 76.29, 80.67, 119.89, 120.54, 126.32, 126.70, 128.03, 128.53, 132.45, 137.78, 142.96, 147.11, 168.34 and 174.88. IR (film-ATR): 3303, 3115, 2960, 2873, 1643, 1603, 1529, 1453, 1410, 1370, 1316, 1259, 1221, 1094, 1043, 973, 831, 753 and 663 cm⁻¹. HRMS (ESI) m/z calcd. for C₃₂H₄₁N₅O₅Cl: 578.2898; found 578.2930 ([M + H]⁺, Δppm 5.5).
207: **4-(2-(trimethylsilyl)ethynyl)pyridine:** 4-Bromopyridine hydrochloride (1.00 g, 5.10 mmol), bis(triphenylphosphine)palladium(II)chloride (0.090 g, 2.5 mol%) and copper iodide (0.024 g, 2.5 mol%) were taken up in diisopropylamine (10 mL) and the mixture was heated to 40 °C. Then, trimethylsilylacetylene (0.800 mL, 5.70 mmol) was added and the mixture immediately turned an opaque black. This was stirred at 40 °C for 12 h. The reaction was quenched with water (10 mL) and CH$_2$Cl$_2$ (5 × 5 mL), dried over MgSO$_4$ and concentrated. The crude, black residue was purified by Kugelrohr distillation (95 °C / 3 mmHg) to give 207 (0.7128 g, 79.7%) as a clear solid. $^1$H NMR (500MHz, CDCl$_3$) δ 0.25 (s, 9H), 7.28-7.30 (m, 2H) and 8.54-8.56 (m, 2H); $^{13}$C (125MHz, CDCl$_3$) δ -0.34, 99.87, 101.93, 125.76, 131.15 and 149.67. IR (film-ATR): 3074, 3039, 2961, 2900, 2166, 2068, 1591, 1573, 1543, 1487, 1406, 1250, 1211, 1067, 989, 862, 842, 819, 760, 701, 644 cm$^{-1}$. HRMS (ESI) m/z calcd. for C$_{10}$H$_{14}$NSi: 176.0896; found 176.0892 ([M + H]$^+$, Δppm 2.0).

208: **4-ethynyl pyridine:** Potassium hydroxide (0.459 g, 8.19 mmol) was added to a solution of 207 (0.7128 g, 4.09 mmol) in MeOH/CH$_2$Cl$_2$ (2:1, 11 mL) and the mixture was stirred at ambient temperature for 2 h, quenched with water (10 mL), extracted with CH$_2$Cl$_2$ (4 × 5 mL), dried over MgSO$_4$ and concentrated. The
crude product was filtered over a silica plug and eluted in CH$_2$Cl$_2$ to give 208 (0.557 g, 30.3%) as a white solid. $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 3.29 (s, 1H), 7.34 (dm, J = 5.87 Hz, 2H), 8.59 (dm, J = 5.87 Hz, 2H); $^{13}$C (125MHz, CDCl$_3$) $\delta$ 80.88, 81.88, 126.04, 130.29, 149.75.

\[ \text{209: 4-phenyl-1-(prop-2-yn-1-yl)piperidine:} \]

4-Phenyl piperidine (0.500 g, 3.10 mmol) was taken up in THF (15.0 mL). Sodium azide (0.067 g, 2.79 mmol) was slowly added and the suspension was stirred at ambient temperature for 2 h. Propargyl bromide (0.4611 g, 3.10 mmol) was added as an 80 % wt. solution in toluene and the mixture was stirred for a further 18 h, during which time it became cloudy. The reaction was quenched with the dropwise addition of water until gas evolution stopped, then filtered over Celite (7 g) which was then rinsed with THF. The filtrate was concentrated and the crude residue was purified by flash chromatography and eluted in EtOAc/Hexanes (1:7) to give 209 (0.3894, 63.0%) as a white, crystalline solid. $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 1.78-1.91 (m, 4H), 2.27 (t, J = 2.2 Hz, 1H), 2.35 (dt, J = 11.7, 2.9 Hz, 2H), 2.50 (m, 1H), 3.02 (dm, J = 11.0 Hz, 2H), 3.36 (m, 2H), 7.18-7.25 (m, 3H), 7.28-7.33 (m, 2H); $^{13}$C (125MHz, CDCl$_3$) $\delta$ 33.39, 42.17, 47.26, 53.01, 72.87, 79.11, 126.16, 126.83, 128.42, 146.17.
210: 1-(5-{1-[4-(pyridin-3-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: Azide 154 (0.0357 g, 0.085 mmol) was taken up in EtOH/H₂O (5:1, 0.500 mL). A 0.5 M solution of 3-ethynyl pyridine (0.340 mL, 0.175 g, 0.170 mmol) in EtOH/H₂O (5:1) was added, followed by a 1.1 M aqueous solution of ascorbate (0.0154 mL, 0.003 g, 0.017 mmol) and a 0.3 M aqueous solution of CuSO₄·5H₂O (0.0283 mL, 0.0014 g, 0.0085 mmol). This mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0154 mL, 0.017 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO₃ which was then rinsed with EtOAc (4 × 2 mL). The filtrate was concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (5:4) to give 210 (0.0133 g, 29.9%). ¹H NMR (500MHz, CDCl₃) δ 0.89 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.4 Hz, 3H), 1.19-1.36 (m, 2H), 1.48 (m, 1H), 1.55 (m, 1H), 1.62-1.70 (m, 2H), 1.70-1.83 (m, 4H), 1.83-1.99 (m, 3H), 2.60 (m, 1H), 2.69 (m, 1H), 3.21 (m, 1H), 3.32 (m, 1H), 4.40 (dd, J = 13.7, 6.4 Hz, 1H), 4.52 (dd, J = 13.7, 3.9 Hz, 1H), 5.11 (m, 1H), 7.21 (dm, J = 8.3 Hz, 2H), 7.27 (dm, J = 8.8 Hz, 2H), 7.37 (m, 1H), 8.01 (s, 1H), 8.29 (dm, J = 7.8 Hz, 1H), 8.57 (m, 1H) and 9.12 (s, 1H); ¹³C NMR (125MHz, CDCl₃) δ 14.58,
20.51, 23.44, 23.50, 29.70, 31.02, 35.73, 35.75, 40.36, 42.21, 53.34, 58.86, 68.97, 75.65, 80.26, 121.61, 123.64, 128.15, 128.33, 132.43, 133.10, 141.96, 144.34, 147.07, 148.88 and 174.91. IR (film-ATR): 3419, 3128, 2965, 2875, 1716, 1490, 1453, 1403, 1236, 1181, 1120, 1093, 1052, 971, 809, 755 and 711 cm\(^{-1}\). HRMS (ESI) \(m/z\) calcd. for \(C_{29}H_{36}N_4O_3Cl\): 523.2476; found 523.2473 ([M + H]\(^+\), Δppm 0.6).

\[
\begin{align*}
\text{Cl} & \quad \text{O} & \quad \text{O} & \quad \text{N}_3 \\
\text{154} & \quad \text{CuSO}_4 \cdot 5\text{H}_2\text{O} & \quad \text{ascorbate} & \quad \text{EtOH/H}_2\text{O} 5:1 \\
\text{Cl} & \quad \text{O} & \quad \text{O} & \quad \text{N}_3 \\
\text{211} & \quad \text{N} & \quad \text{N} & \quad \text{N}
\end{align*}
\]

211: 1-(5-{1-[4-(pyridin-4-yl)-1H-1,2,3-triazol-1-yl]propan-2-yl}oxolan-2-yl)propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate: Azide 154 (0.033 g, 0.0785 mmol) was taken up in EtOH/H\(_2\)O (5:1, 0.500 mL). A 0.5 M solution of 4-ethynyl pyridine 208 (0.314 mL, 0.0162 g, 0.157 mmol) in EtOH/H\(_2\)O (5:1) was added followed by a 1.1 M aqueous solution of ascorbate (0.0142 mL, 0.0028 g, 0.0157 mmol) and a 0.3 M aqueous solution of aqueous solution of CuSO\(_4\)•5H\(_2\)O (0.026 mL, 0.0013 g, 0.0079 mmol). The mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0142 mL, 0.0157 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO\(_3\) which was then rinsed with EtOAc (4 × 2 mL). The filtrate was
concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (5:4) to give 211 (0.0123 g, 29.9%). $^1$H NMR (500MHz, CDCl$_3$) $\delta$ 0.90 (d, J = 6.8 Hz, 3H), 1.13 (d, J = 6.4 Hz, 3H), 1.19-1.34 (m, 2H), 1.44-1.57 (m, 2H), 1.59-1.96 (m, 9H), 2.61 (m, 1H), 2.70 (m, 1H), 3.15 (m, 1H), 3.23 (m, 1H), 4.45 (dd, J = 13.7, 5.9 Hz, 1H), 4.50 (dd, J = 13.7, 3.9 Hz, 1H), 5.15 (m, 1H), 7.21 (dm, J = 8.8 Hz, 2H), 7.26 (dm, J = 8.8 Hz, 2H), 7.88 (d, J = 5.4 Hz, 2H), 8.13 (s, 1H), 8.66 (d, J = 4.9 Hz, 2H); $^{13}$C NMR (125MHz, CDCl$_3$) $\delta$ 14.63, 20.56, 23.43, 23.51, 29.79, 31.00, 35.69, 35.76, 40.31, 42.18, 53.29, 58.90, 68.90, 75.57, 80.07, 120.08, 123.08, 128.17, 128.29, 132.48, 138.49, 141.92, 144.80, 150.14, 175.03. IR (film-ATR): 3130, 2962, 2874, 1716, 1611, 1562, 1491, 1455, 1383, 1237, 1187, 1120, 1092, 1017, 826, 756 and 690 cm$^{-1}$. HRMS (ESI) $m/z$ calcd. for C$_{29}$H$_{36}$N$_4$O$_3$Cl: 523.2476; found 523.2483 ([M + H]$^+$, $\Delta$ppm 1.3).

212: 1-[5-(1-{4-[4-phenylpiperidin-1-yl]methyl}-1H-1,2,3-triazol-1-yl)propan-2-yl]oxolan-2-yl]propan-2-yl 1-(4-chlorophenyl)cyclopentane-1-carboxylate:

Azide 154 (0.0332 g, 0.079 mmol) was taken up in EtOH/H$_2$O (5:1, 0.500 mL). A 0.5 M solution of N-ethynyl-4-phenyl piperidine 209 (0.316 mL, 0.0315 g, 0.158 mmol) in EtOH/H$_2$O (5:1) was added, followed by a 1.1 M aqueous solution of
ascorbate (0.0143 mL, 0.0028 g, 0.0158 mmol) and a 0.3 M aqueous solution of CuSO$_4$•5H$_2$O (0.0263 mL, 0.0013 g, 0.0079 mmol). The mixture was shaken at 225 RPM at 37 °C for 14 h. The reaction was still not complete as determined by TLC (EtOAc/Hexanes, 1:1) so more 1.1 M ascorbate (0.0143 mL, 0.0158 mmol) was added and the mixture was shaken for a further 24 h. The mixture was filtered over a plug of solid NaHCO$_3$ which was then rinsed with EtOAc (4 × 2 mL). The filtrate was concentrated and purified by flash chromatography and eluted with EtOAc/Hexanes (5:4) to give 212 (0.0123 g, 29.9%). $^1$H NMR (500MHz, CDCl$_3$) δ 0.81 (d, J = 6.8 Hz, 3H), 1.14 (d, J = 6.4 Hz, 3H), 1.31 (m, 1H), 1.47 (m, 1H), 1.58 (ddd, J = 14.2, 8.8, 4.4 Hz, 1H), 1.66 (m, 1H), 1.70-1.78 (m, 5H), 1.78-1.93 (m, 8H), 1.96 (m, 1H), 2.20 (br s, 2H), 2.49 (m, 1H), 2.59 (m, 1H), 2.68 (m, 1H), 3.10 (d, J = 10.8 Hz, 2H), 3.28 (m, 1H), 3.42 (m, 1H), 3.77 (s, 2H), 4.22 (dd, J = 13.7, 7.8 Hz, 1H), 4.51 (dd, J = 13.7, 3.9 Hz, 1H), 5.00 (m, 1H), 7.16-7.23 (m, 3H), 7.25 (m, 1H), 7.26-7.28 (m, 2H), 7.28-7.32 (m, 3H), 7.57 (br s, 1H); $^{13}$C NMR (125MHz, CDCl$_3$) δ 14.35, 20.43, 23.46, 23.53, 29.45, 31.08, 33.25, 35.81, 35.86, 40.30, 42.22, 42.40, 53.22, 53.55, 53.94, 58.80, 69.27, 75.77, 80.60, 126.09, 126.84, 128.19, 128.34, 128.37, 132.41, 142.02, 174.75. IR (film-ATR): 3138, 3027, 2934, 2874, 1720, 1602, 1492, 1454, 1379, 1324, 1236, 1095, 1050, 912, 829, 754 and 701. HRMS (ESI) m/z calcd. for C$_{36}$H$_{48}$N$_4$O$_3$Cl: 619.3415; found 619.3430 ([M + H]$^+$, Δppm 2.4).
CHAPTER 10

BIOLOGICAL EXPERIMENTALS
10.1 General Procedures

*Saccharopolyspora spinosa* was purchased from ATCC and used for the following genetic manipulations. Media components were purchased from VWR/Difco and Sigma. Vegetative media consisted of 30 g soytone, 3 g yeast extract, 3 g magnesium sulfate heptahydrate, 5 g glucose and 5 g maltose per L DI water. Production I media consisted of 40 g glucose, 30 g vegetable peptone, 3 g calcium carbonate and 10 g tryptic soy broth (TSB) per liter of tap water. TSB broth consisted of 30 g TSB per liter of DI water. ABB13 media consisted of 30 g soytone, 5 g soluble starch, 3 g calcium carbonate, 2.1 g MOPS, and 20 g agar (when appropriate) per L of DI water, following autoclaving for 20 min at 125 °C, 1 mL thiamine HCl and 1-2 mg of iron(II) sulfate are added. LB broth consisted of 10 g tryptone, 5 g NaCl, and 5 g yeast extract per L DI water. NZI+ broth consisted of 10 g NZ amine, 5 g yeast extract, 5 g NaCl per liter DI water. After autoclave, add 12.5 mL of 1M MgCl₂ and 12.5 mL of 1M MgSO₄ and 10 mL of 2M glucose. Primers were purchased through Integrated DNA Technologies and diluted to a working concentration of 10 pmol/μL in nanopure water. HotMaster Taq DNA polymerase was purchased from 5 Prime and was used for all PCR reactions. PCR reactions were performed on a Strategene Thermocycler. Restriction endonucleases were purchased from Promega. *Escherichia coli* DH5α and non-methylating strains ET12567 and ET12567/pUZ8002 were routinely used for DNA cloning. The plasmid pUZ8002 encodes for the necessary cellular machinery to transfer transmissible plasmids to a recipient bacteria. *E. coli* carrying pGEM derivatives were grown in LB
containing carbenicillin (25 μg/mL) and *E. coli* carrying pOJ260 derivatives were grown in LB containing apramycin (50 μg/mL). Any water used in restriction digests or PCR reactions is nanopure. Electroporations were performed on a BioRad Micropulser in 0.2 cm electroporation cuvettes. All plasmids produced were verified by DNA sequencing by the Murdock Lab at the University of Montana.

### 10.2 Alamar Blue Assay

Compounds to be tested were dissolved in DMSO to a concentration of 50 mM. One hundred microliters of solution were placed in the top row of a 96 well plate and stock plates of serial dilutions of the compounds were made by taking 50 μL from the top row and diluting it by with 50 μL of fresh DMSO in the second row. This process was repeated down the plate until each column had a concentration range of 50 mM to 0.39 mM, which, when only using 2 μL from each well for the assays correspond to actual assay concentrations of 1000 μM – 8 μM. When performing an assay, 2 μL of solution from the serial dilution plate were used to make replica plates for use in the assay. Organisms that were being tested against were grown in appropriate media overnight. In the morning, the cultures were diluted to an O.D.₆₀₀ of 0.2 with fresh media. For some organisms, it was necessary to dilute further to attain approximately $10^5$ organisms in each well for the assay. This extra dilution factor is determined in the lab for each stock of organism used. Generally speaking, however, a 100-fold dilution from the original overnight culture is sufficient to get to a working assay concentration. Once the dilution was prepared, 100 μL of the dilute culture was aliquoted into each well and
the plate was incubated for several hours. The exact times and temperatures again are
dependent upon the particular organism being tested against. After the incubation, 10
µL of a 0.03 % w/v solution of resazurin in water that had been sterilized by filtration
through a 0.2 µM filter was added to each well. After a short time (5 – 30 min), a
colorimetric change is visible and the first concentration at which a different color is
apparent is recorded as the MIC value of that particular compound for that particular
organism (Figure 2.10).

10.3 Extraction of Genomic DNA from *Saccharopolyspora spinosa*

150 mL of TSB broth was inoculated with 150 µL of a culture of *S. spinosa*. The
culture was incubated at 30 °C at 180 RPM on a shaker for 2 d. The culture was
transferred to a falcon tube and centrifuged at 3000 × g for 10 min. The supernatant
was decanted and the pellet was re-suspended in 10% sucrose and centrifuged again at
4000 × g for 5 min. The supernatant was again decanted and the pellet resuspended in
5 mL lysis solution (0.3 M sucrose, 25 mM EDTA, 25 mM Tris-HCl). Lysozyme (≈ 3 mg)
was added and the mixture was incubated at 35 °C for 30 min. 1 mL of 10% SDS was
added followed by proteinase K (≈ 3 mg) and the mixture was incubated at 55 °C for 1 h
with occasional inversions. Chloroform (3.6 mL) was added and the tube was inverted
constantly at ambient temperature for 30 min. The cloudy mixture was then
centrifuged at 5000 × g for 20 min. The top layer was carefully transferred to a clean
tube and the bottom layer was discarded, isopropanol was gently added and the tube
was inverted once. The genomic DNA was then spooled and placed in a microcentrifuge

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tube, then rinsed with 70% EtOH. The EtOH was dried and the DNA was redissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA).

10.4 PCR Amplification of the spnB gene

The DNA primers “spn28916_Xbal(F)”: 5’ – GATTAGTTCTAGAATGACGG TGACCACCAGTTACGAAGAAGTTG – 3’ and “spn35374_PstI(R)”: 5’ – GATTA GTCTGCAGTCAGGGAAGTTCGAGATCGTTGTCGAGGAGT – 3’ were used to amplify the spnB gene and concurrently introduce Xbal and PstI restriction sites for eventual cloning into pOJ260. 10× buffer (2 μL), 25 μM dNTPs (4 μL), DMSO (2 μL), primers (1 μL forward, 1 μL reverse), S. spinosa genomic DNA (0.8 μL), Taq polymerase (0.6 μL), and water (8.6 μL) were mixed in a PCR tube and the following temperature program was run: 1) 95 °C / 2 min, 2) 95 °C /30 s; 60 °C /40 s; 65 °C / 7 min for 31 cycles and 3) 65 °C / 10 min. The finished PCR product was purified on a 0.8% agarose gel and the band at 6.4 kb was excised using a DNA Extraction Kit (QIAGEN).
10.5 Ligation of *spnB* into pGEM-T EZ [pWS3500]

![Diagram of plasmid with restriction sites](image)

**Figure 10.1: Plasmid pWS3500.** A p-GEM-T EZ derived plasmid containing a full copy of the *spnB* gene.

The *spnB* gene was ligated into pGEM-T EZ according to manufacturer’s instructions and grown in *E. coli* XL-10 cells provided in the kit. The resultant colonies that grew were screened by restriction digest of their produced plasmids. Each colony to be screened was grown up in 2 mL LB media containing ampicillin (25 μg/mL) overnight at 37 °C at 225 RPM on a shaker in round bottomed 15 mL Falcon tubes (RB Falcon). The cultures were centrifuged the next morning at 6000 × g for 6 min. The media was discarded and the plasmids were extracted using a Mini Prep Kit (QIAGEN). The plasmids are dissolved in TE buffer at the end of the purification process. The digestions were carried out as follows: 10× buffer H (1 μL), 10× Bovine Serum Albumin (BSA) (1 μL), plasmid (5 μL), water (2.5 μL) and PstI or XbaI (0.5 μL). Once the correct plasmid was found it was electroporated into *E. coli* DH5α.
**General electroporation procedure:** 50 μL of electrocompetent DH5α cells were thawed from -80 °C, 2.5 μL of plasmid were added and mixed by pipetting. The cells were placed in the cuvette and pulsed (1.8 mV, 5.5 ms). They were then taken up in 1 mL LB broth, transferred to an RB Falcon and incubated for 1 h at 37 °C at 225 RPM. 200 μL of this culture was then spread onto LB agar plates containing carbenicillin (25 μg/mL) and incubated overnight at 37 °C.

### 10.6 Mutagenesis of pWS3500 to pWS3501 by addition of a BclI site

![Diagram of p-GEMT EZ derived plasmid](image)

**Figure 10.2:** Plasmid pWS3501. A p-GEMT EZ derived plasmid containing the *spnB* gene with an engineered BclI restriction site. The naturally occurring BclI site is in black on the left; the engineered site is in purple on the right.

In order to eventually introduce the hyg<sup>R</sup> sacB cassette into the *spnB* gene, a second BclI site had to be engineered into *spnB*. Using the Stratagene Quickchange II XL Site Directed Mutagenesis Kit, the primers “spnB_5278_BclI(F)” 5’ – GGTCTCTCGTACGCGCCTGATCACGCGCCAGTGGA – 3’ and “spnB_5278_BclI(R)” 5’ – CCACTGGGCTGCGTGATCAAGCCCTGCGTGACGAGGACC – 3’ (restriction sites in bold and
underlined, mutations are red) were used to introduce another BclI site into pWS3500 (new site on right in figure above). 10× Reaction Buffer (5 μL), pWS3500 (1 μL), primers (1.2 μL F; 1.2 μL R), dNTP mix from kit (1 μL), Quik Solution from kit (3 μL), water (37.6 μL) and Pfu Turbo Polymerase from kit (1 μL). The PCR temperature program was as follows: 1) 95 °C / 1 min 2) 95 °C /50 sec ; 60 °C / 50 sec ; 68 °C / 10 min for 18 cycles 3) 68 °C / 7 min. After the program completes, 1 μL (10 units) of DpnI was added to the PCR tube, mixed and incubated at 37 °C for 1 h. The DNA was then introduced into chemically competent E. coli XL-10 gold cells via heat shock using the protocol provided by the manufacturer, plated on LB plates containing carbenicillin (25 μg/mL) and incubated overnight. Plasmids were then extracted from the resultant colonies using the procedure described above and electroporated into the non-methylating E. coli strain ET12567. This is because BclI is inhibited by DNA methylation and will not cut DNA from a methylating strain such as XL-10, making screening difficult. The proper plasmid was found by restriction digest screening with BclI and named pWS3501.
10.7 Mutagenesis of pWS3501 to pWS3502 by the introduction of the Serine/Proline mutations and concurrent introduction of a new BstXI restriction site

![Diagram of plasmid pWS3502 with annotations](image)

**Figure 10.3:** Plasmid pWS3502. A p-GEMT EZ derived plasmid with an engineered mutation in the active site of the ER domain. A BstXI site is concurrently engineered by the ER domain inactivation.

The primers “spnB_4648_ER_serpro_BstXI(F)” 5’ – CATTCGGCGCCAGTCCAGTGGGGATGGCC – 3’ and “spnB_4648_ER_serpro_BstXI(R)” 5’ – GCCATCCCCACTGGACTGGCGGCGGAATG – 3’ (mutations shown in red, serine/proline site bold and underlined) were used to introduce the mutations to change the two adjacent glycine residues in the NADPH binding site into a serine and a proline. The mutagenesis was performed as described above for the mutagenesis of the extra BclI site except for the primers used. Plasmids were extracted from the resultant colonies and screened by restriction digest with BstXI. The correct plasmid was introduced into DH5α via electroporation and named pWS3502.
10.8 Subcloning the spnB Cassette into pOJ260

Figure 10.4: Plasmid pWS3503. A pOJ260-derived plasmid containing the engineered, inactivated spnB gene.

In order to be able to transfer to the S. spinosa from an E. coli donor by conjugal transfer, the altered spnB gene must be in a transferrable plasmid, which means that it needs to be moved to pOJ260 from pGEM-T EZ. The PstI and XbaI sites that were introduced when spnB was cloned will be used for a sticky end ligation into complementary restriction sites in pOJ260. First, both the spnB cassette (insert) and pOJ260 (vector) need to be digested with the same restriction enzymes. Plasmid pWS3502 and pOJ260 were both prepared from ET12567 hosts with the Mini Prep Kit (QIAGEN) and digested as follows: Plasmid (16.8 µL), BSA (0.2 µL), 10× buffer H (2 µL), PstI (0.5 µL) and XbaI (0.5 µL) were mixed and incubated for 3 h at 37 °C. The resultant bands were excised and purified using the DNA extraction kit (QIAGEN) as before and used for the ligation reaction: Insert (3 µL), Vector (6 µL), water (4.7 µL), 10× Ligation Buffer (1.6 µL) (provided with purchase of T4 DNA Ligase) and T4 DNA Ligase (0.7 µL) were mixed by pipetting in a ligation tube and incubated at ambient temperature for 3
h. Immediately after incubation, the ligation mixtures were used to transform chemically competent XL-10 cells by heat shock.

**Transformation of XL-10 cells:** An aliquot (100 μL) of XL-10 cells were thawed from -80 °C on ice and transferred to a chilled RB Falcon. β-mercaptoethanol (4 μL) was added and the mixture was incubated on ice for 10 min with swirling every 2 min. 2 μL of ligation mixture was added and mixed with gentle swirling, this was incubated on ice for 30 min. The tube was then heat shocked in a water bath at 42 °C for 30 sec and immediately put back in the ice for 2 min. Preheated (42 °C) NZY+ broth (0.9 mL) was added to the tube and the cells were incubated at 37 °C at 225 RPM in a shaker for 1 h. 200 μL of the culture were plated onto LB plates containing apramycin (50 μg/mL) and incubated overnight at 37 °C. Plasmids extracted from the resultant colonies were screened by restriction digest as before, this time using PstI and Xbal. The proper plasmid was introduced into E. coli ET12567/pUZ8002 via electroporation for eventual bacterial conjugation and named pWS3503.
10.9 Ligation of hyg$^R$ sacB cassette into pWS3503

![Diagram](image.png)

**Figure 10.5:** Plasmid pWS3503. A pOJ260-derived plasmid containing a *spnB* gene truncated with a hygromycin resistance and sucrose sensitivity cassette.

The first round of homologous recombination will be done in order to replace the wild type *spnB* gene with hygromycin resistance and sucrose sensitivity markers. The BclI sites from pWS3503 are complementary to the flanking BamHI sites on the hyg$^R$ sacB cassette residing in plasmid pAS406 made by Smith. The hyg$^R$ sacB cassette (insert) was cut out from pAS406 by digestion with BamHI and PstI:  10× Buffer H (2 μL), BSA (0.2 μL), pAS406 (16.8 μL), BamHI (0.5 μL) and PstI (0.5 μL) were mixed and incubated at 37 °C for 3 h. Plasmid pWS3502 (vector) was digested with BclI:  10× buffer C (2 μL), BSA (0.2 μL), pWS3503 (17.3 μL) and BclI (0.5 μL) were mixed and incubated at 50 °C for 3 h. After this incubation was gel purified, 20 μL of the plasmid was transferred to a new microcentrifuge tube, 2.5 μL of 10× SAP buffer and 2.5 μL Shrimp Alkaline Phosphatase (SAP) were added and the mixture was incubated at 37 °C for 1 h. The SAP treatment is to ensure that the complementary BclI sites do not re-ligate when attempting to insert the hyg$^R$ sacB cassette. The ligation was then preformed as follows: Insert (8 μL), Vector (8 μL), water (1.3 μL), 10× T4 Ligase buffer (2 μL) and T4 DNA Ligase (0.7 μL) were added to a ligation tube and incubated at ambient temperature for 1 h then at 4 °C.
overnight. The next day, the ligation mixture was used to transform chemically competent XL-10 cells using heat shock as described above. 250 µL of the culture was plated onto LB plates containing apramycin (50 µg/mL) and incubated at 37 °C overnight. The plasmids from the resultant colonies were screened by restriction digest with EcoRI, which indicated both presence and orientation of the inserted cassette. The correct plasmid was found and introduced into *E. coli* ET12567/pUZ8002 via electroporation and named pWS3503Ω.
References


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APPENDIX: SELECTED NMR SPECTRA FOR SYNTHESIZED COMPOUNDS
4: $^{13}$C-NMR
5: $^1$H NMR
6: $^1$H NMR
6: $^{13}$C NMR
7: $^1$H NMR
8: $^{13}$C NMR
40: $^1$H NMR
$^{13}$C NMR
53: $^1$H NMR
53: $^{13}\text{C}$ NMR
53: $^1$H-$^1$H COSY
53: $^{13}$C-^1^H HETCOR
56: $^1$H NMR
56: $^{13}$C NMR
$^{1}H$ NMR
57: $^{13}$C NMR
59: $^1$H NMR
60: $^{13}$C NMR
61: $^1$H NMR
62: $^{13}$C NMR
63: $^{13}$C NMR
64: $^{13}\text{C NMR}$
88: $^1$H NMR
89: $^1$H NMR
90: $^1$H NMR
91: $^1$H NMR
91: $^{13}$C NMR
92: $^1$H NMR
SAR-2A-5

Pulse Sequence: s2pul
Solute: CDCl3
Ambient temperature
UNITYplus-460 "gyro"

Pulse, delay 2.380 sec
Pulse 60.2 degrees
Eq. time 4.860 sec
Width 2274.4 Hz
NMR repetitions
Observe CE3, 188.5076667 MHz
Decouple H1, 199.306333 MHz
Power 89 dB
continuously on

Data Processing
Lin. broadening 1.0 Hz
FT size 32768
Total time 9 hr, 1 min, 31 sec

92: $^{13}$C NMR
93: \textsuperscript{1}H NMR
94: $^1$H NMR
96: $^{13}$C NMR
100: $^1$H NMR
SAM-2A-22

Pulse Sequence: s2pul
Solvent: DDCi3
Ambient temperature: 300 K
UNITYplus-490 "pyro"

Delay, delay 2.000 sec
Pulse 90.2 degrees
Acq, time 0.005 sec
Width 22714.6 Hz
310 C repetitions

Observe C13, 100.5626667 MHz
Decoupled H1, 399.3526330 MHz

FT, continuously on
MULT1-16 modulated
DATA PROCESSING

Line broadening 1.0 Hz
FT size 20768
Total time 8 hr, 20 min, 33 sec

100: $^{13}$C NMR
101: $^1H$ NMR
SAR-2A-26
Pulse Sequence: G2pul
Solvent: CDC13
Ambient temperature
UNITYplus-400 “gyro”
Pulse, delay 3.500 sec
Pulse 60.2 degrees
Exc. time 0.025 sec
Width 22744.4 Hz

Observe C13, 100.5076667 MHz
DECOUPLE H1, 399.9506330 MHz
Power 30 dB
Continuous on

DATA PROCESSING
Lin. broadening 1.0 Hz
FT size 32768
Total time 4 hr, 44 min, 24 sec

101: $^{13}$C NMR
102: $^1$H NMR
SAR-IA-27

Pulse Sequence: szpul
Solute: CDCl3
Ambient temperature
0.5Hplus-400 "gyro"

Relax. delay 3.000 sec
Pulse 60.2 degrees
Acq. time 0.285 sec
Width 2716.4 Hz
4608 repetitions

OBSERVE G30, 300.6276667 MHz
DECOUPLE G1, 300.627630 MHz
Power 40 dB
continuously

DATA PROCESSING
Line Broadening 3.6 Hz
FT nter 32768
Total time 4 hr, 44 min, 24 sec

102: $^{13}$C NMR
162: $^1$H NMR
163: $^1$H NMR
164: $^1$H NMR
165: $^1$H NMR
166: $^1$H NMR
167: $^1$H NMR
169: $^1$H NMR
171: $^1$H NMR
172: $^1$H NMR
174: $^1$H NMR
174: $^{13}$C NMR
176: $^1$H NMR
195: $^1$H NMR
196: $^1$H NMR
$^{1}H$ NMR
202: $^{13}$C NMR
203: $^1$H NMR
203: $^{13}$C NMR
204: $^1$H NMR
206: $^{13}$C NMR
208: $^1H$ NMR
210: $^1$H NMR
210: $^{13}$C NMR
212: $^{13}$C NMR