Arresting the Spliceosome

Investigations into the role of Snu114 within the spliceosome

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy

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Abstract

Spicing is the process where pre-mRNA is converted to mRNA via two transesterification reactions. With this process unwanted sequences of nucleic acids, known as introns, are removed allowing only the coding nucleic acid sequences, exons, to remain. This process is catalysed by a dynamically assembled, highly complex macromolecular machine called the spliceosome, which is made up of five small nuclear ribonucleoproteins (snRNPs). To date, the spliceosome has defied conventional methods for conclusive characterisation, resulting in it being relatively poorly understood, although advances have been made.\textsuperscript{1,2} Apart from being of interest due to the fact that splicing is an essential life process, it is also of interest medically. Disruption to the splicing process can produce incorrectly formed mRNA, which plays a part in many diseases.\textsuperscript{3} Small molecule inhibitors which bind to, and inhibit, the functions of individual proteins would “stall” the spliceosome,\textsuperscript{4} circumventing its dynamic nature. These inhibitors could also form the basis of new drugs, treating diseases which incorrectly formed mRNA can cause.

Previously reported small molecule inhibitors have inhibited splicing at the early stages of spliceosome assembly.\textsuperscript{5-7} However, our target protein snu114\textsuperscript{8} belongs to the U5 snRNP, which is involved later on in the splicing cycle. Inhibition of Snu114 should, therefore, lead to accumulation of spliceosome complexes produced at later stages of the cycle. Homology studies of Snu114 indicated a strong correlation of amino acid sequences with ribosomal growth factors EF-2 and EF-G. This study allowed us to target Snu114 using known EF-2 and EF-G inhibitors, sordarin and fusidic acid, which were tested and found to have significant splicing inhibition activity. A series of derivatives of these parent compounds were then attempted in an effort to improve splicing inhibition activity and to analyse the structure-activity relationship of fusidic acid and sordarin as splicing inhibitors. The biosynthesis of sordarin proved to be difficult and only a few derivatives were synthesised, however an improvement was made to splicing inhibition activity by forming sordaricin 32. Various fusidic acid derivatives were successfully synthesised, leading to an analysis of the structure-activity relationship of fusidic acid as a splicing inhibitor. Most fusidic acid derivatives produced a lower splicing inhibition activity than fusidic acid. However, fusidic acid derivative 229 had an equivalent inhibition activity to that found for fusidic acid. This result leads us to believe that the C-3 hydroxyl moiety of fusidic acid would be an ideal area for modification in future studies.
Declaration
No portion of this thesis has been submitted in support of an application for another degree or qualification of this or any other university, or any other institute of learning.

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**Abbreviations**

[α]  Specific rotation  
Δ  Heated  
δ  Parts per million  
A  Adenosine  
Ar  Aryl  
ATP  Adenosine triphosphate  
BA3  Butyrolactone 3  
Bn  Benzyl  
Boc  tert-butoxycarbonyl  
Bu  Butyl  
cat.  Catalytic  
Cbz  Benzoyloxycarbonyl  
COSY  Correlation spectroscopy  
d  doublet  
DACH  1,2-Diaminocyclohexane  
DAST  N,N-diethylaminosulfur trifluoride  
DBU  1,8-Diazabicycloundec-2-ene  
DCM  Dichloromethane  
DDQ  2,3-Dichloro-5,6-dicyano-1,4-benzoquinone  
DEPT  Distortionless Enhancement by Polarization Transfer  
DHC  Dihydrocoumarin  
DIAD  Diisopropyl azodicarboxylate  
DIBAL  Diisobutylaluminium hydride  
DMAP  4-dimethylaminopyridine  
DMF  N,N-Dimethylformamide  
DMP  2,2-Dimethoxypropane  
DMS  Dimethyl sulfide
DMSO  Dimethyl sulfoxide
DNA  2’-Deoxyribonucleic acid
EDA  Ethylenediamine
ee  enantiomeric excess
EJC  Exon junction complex
ES  Electrospray
ESS  Exonic splicing silencer
EWG  Electron withdrawing group
G  Guanine
GTP  Guanosine-5’-triphosphate
GDP  Guanosine-5’-diphosphate
h  Hours
HAT  Histone acetyltransferases
HDAC  Histone deacetylases
HIV  Human immunodeficiency virus
HMPA  Hexamethylphosphorictriamide
HMPT  Hexamethylphosphorustriamide
HMQC  Heteronuclear single-quantum correlation spectroscopy
HPLC  High performance liquid chromatography
HOMO  Highest occupied molecular orbital
IC_{50}  half maximal inhibitory concentration
Im  Imidazole
i-Pr  iso-propyl
IR  Infrared spectroscopy
L  litre
LC  Liquid chromatography
LC-MS  Liquid chromatography mass spectrometry
LDA  Lithium diisopropylamide
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<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
</tr>
<tr>
<td>M</td>
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<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>Me</td>
<td>Methyl</td>
</tr>
<tr>
<td>mCPBA</td>
<td>meta-chloroperbenzoic acid</td>
</tr>
<tr>
<td>MOM</td>
<td>Methoxymethyl</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acid</td>
</tr>
<tr>
<td>ms</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NBS</td>
<td>N-Bromosuccimide</td>
</tr>
<tr>
<td>NMO</td>
<td>N-Methylmorpholine-N-oxide</td>
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<tr>
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<tr>
<td>snRNA</td>
<td>Small nuclear ribonucleic acid</td>
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<tr>
<td>snRNP</td>
<td>Small nuclear ribonucleoproteins</td>
</tr>
<tr>
<td>SR proteins</td>
<td>Serine rich proteins</td>
</tr>
<tr>
<td>t</td>
<td>Triplet</td>
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<td>TADA</td>
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</tr>
<tr>
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<td>para-toluene sulfonic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Extract Peptone Dextrose</td>
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1.0 Introduction

1.1 The Central Dogma of Molecular Biology

The field of molecular biology emerged in the decades following the publication of the helical structure of DNA. The “Central Dogma” of molecular biology was first described by Francis Crick and outlines how genetic information is transferred in a single direction through a biological system. In this process, the information encoded within a gene is copied from DNA into RNA via transcription and from RNA into protein via translation. Although essentially correct, it failed to anticipate special exceptions discovered subsequently, such as reverse transcription and RNA replication (Figure 1).

![Figure 1: Schematic outline of the flow of information between nucleic acids and protein. Unusual flow of information highlighted by dashed arrows.](image)

1.2 Transcription

Transcription is the process by which RNA is produced, which is complementary in sequence to the parental DNA template strand. The process is achieved in three major steps (Figure 2).

The first of these steps is called initiation, which is where the transcription complex is formed on a promoter sequence upstream of the gene and the two DNA strands ‘melt’ allowing access to the template strand. In eukaryotes, a pre-initiation complex must be
formed upstream of the gene which is to be transcribed, before initiation can take place. This takes place at a special site called the core promoter sequence, which provides specific binding sites for transcription factors, and eventually the RNA polymerase. The transcription factors are ancillary proteins, which serve to recognise the promoter, bind and align the RNA polymerase correctly and help ‘melt’ the DNA duplex. RNA polymerase itself is the core enzyme which actually performs transcription. In eukaryotes three different RNA polymerases exist, termed RNAPs I, II and III. RNAP I transcribes mainly ribosomal RNA genes, RNAP II transcribes protein genes and most URNA genes, whilst RNAP III transcribes genes for short RNAs such as the tRNA genes. Various sites around the promoter bind activator and repressor proteins, which are responsible for modulating the rate of transcription initiation. The core promoter contains a special sequence called the TATA box (5’-TATAAA-3’), which is recognised by transcription factors and is the initial site which the DNA duplex is melted. In bacteria the preinitiation complex is unnecessary, as the RNA polymerase binds directly to the core promoter sequence via a special subunit of the enzyme called the sigma (σ) factor.

![Figure 2: Eukaryotic DNA transcription.](image)

Once the RNA polymerase is in position and the first bond forms between ribonucleotides in the active site, the elongation step occurs. In this process RNA
polymerase traverses one of the DNA strands known as the “template strand” or “non-coding strand” and uses base pairing complementarity with the DNA template to create a single stranded RNA copy. This RNA copy is actually a direct copy of the “coding strand” as the RNA is transcribed to be complementary to the template strand upon which it is polymerised. The RNA polymerase migrates along the template strand, melting and unwinding the helix. Behind the RNA polymerase the duplex reforms so that the ‘transcription’ bubble encompasses only 12-17 nucleotides of the DNA template strand at a time.

The third and final step, termination is well defined in bacteria and is achieved in one of two ways, the termed Rho-dependent and Rho-independent transcription termination. Rho-independent transcription termination occurs when the newly synthesised RNA molecules forms a G-C-rich loop followed by a series of uridine residues. This stem loop structure is thought to interact with the transcribing RNA polymerase causing it to pause. This allows the relatively weak A-U pairs of the DNA-RNA duplex to break and the RNA is pulled out of the active site of the RNA polymerase, terminating transcription. Rho-dependent transcription termination occurs when a protein factor called “Rho” binds to the RNA at a specific site, migrates along the transcript until it reaches the RNA polymerase. Once this migration is complete the Rho protein factor destabilizes the interaction between the template sequence and the RNA, terminating transcription. In eukaryotes the termination step is still not well understood. It is possible that no specific termination signal exists. Alternatively the RNA transcript may fold in such a way that disrupts the RNA polymerase causing the enzyme to ‘fall off’ the DNA template thereby terminating transcription. Study of transcription termination is complicated by the fact that the 3’-end of the nascent transcript is cleaved off prior to the attachment of the polyadenosine tail.

1.3 Translation
Translation is the process by which information contained within the nucleotide sequence of a messenger RNA (mRNA) produced via transcription, is decoded in the ribosome to produce the specific amino acid chain required for a particular protein (Figure 3). The ribosome is a complex multisubunit RNA-protein complex responsible for catalysing
peptide bond formation during the translation process.\textsuperscript{23} Two other main components take part in the process of translation, mRNA and transfer RNAs (tRNAs).\textsuperscript{24} tRNAs are a particular type of RNA, which are short (typically <100 nucleotides long), ‘charged’ with an amino acid at the 3’-end by specific enzymes tRNA aminoacylases, and which contain a specific 3 nucleotide sequence which is complementary to a sequence of three nucleotides in the mRNA known as a codon.

\textbf{Figure 3:} Translation of RNA into Protein.

In common with transcription, the process of translation occurs in three main stages, initiation, elongation and termination. The first stage, initiation is where the small subunit of the ribosome binds to the 5’-end of the mRNA, with the help of proteins known as initiation factors, and locates the start codon (AUG), where a specific initiator tRNA binds to initiate translation. In prokaryotes a ribosome binding site exists in which a specific sequence (the Shine-Dalgarno sequence) base pairs with the RNA component of the small ribosomal subunit. In eukaryotes, the 5’-cap structure is recognised allowing the small ribosomal subunit to scan the mRNA until it reaches the start codon. Once the initiator tRNA is in place the large ribosomal subunit binds, and the elongation phase begins.
During elongation, a specific tRNA enters a cleft in the ribosome called the aminoacyl (A) site, and base pairs specifically with the codon in the mRNA. An ancillary protein EFTu serves a proof-reading role, confirming that the correct tRNA is in place, then peptide bond formation takes place between the amino acid in the A-site and the peptide attached to a tRNA in the adjacent peptidyl (P)-site. The ribosome then moves along one codon in a translocation step, which is caused by a change in the conformation of the ribosome induced by the hydrolysis of GTP. Translocation ejects the uncharged tRNA from the ribosome and the tRNA carrying the peptide chain enters the P-site. This process continues with amino acids being covalently attached together, polymerising into a specific polypeptide sequence dictated by the particular mRNA sequence.

Termination of the polypeptide sequence occurs when the ribosome reaches a stop codon. A stop codon is a codon for which there is no complimentary tRNA. Instead of elongation, a protein known as ‘a Release Factor’ enters the A-site and promotes the disassembly of the ribosome/mRNA complex.

1.4 Pre-mRNA Processing

In most prokaryotes, mRNA is essentially mature after transcription and requires no processing. However in eukaryotes, post transcription pre-mRNA must undergo extensive processing to turn it into mature mRNA, ready for translation. This processing takes the form of 5’-capping, 3’-polyadenylation and splicing (see Section 1.5).

![Figure 4: Structure of the 5’-cap of pre-mRNA](image-url)
5’-Capping involves the formation of a specially altered nucleotide on the 5’-end of pre-mRNA (Figure 4). The 5’-cap has multiple functions, including regulation of nuclear export, prevention of degradation and the promotion of the subsequent translation and splicing processes.\textsuperscript{25-27} The 5’-capping process starts by cleaving one of three terminal phosphates \textit{via} RNA terminal phosphatase. This cleavage is followed by the addition of guanosine-5’-triphosphate (GTP) \textit{via} guanylyltransferase. GTP loses two phosphates in the process thus forming the 5’,5’-triphosphate bridge. The N-7 atom of guanine is methylated by a methyltransferase forming 7-methylguanosine, and hence the 5’-cap.

3’-Polyadenylation is the addition of a poly(A) tail onto the 3’-end of pre-mRNA, and consists of multiple adenosine monophosphates. The poly(A) tail aids in the protection of the pre-mRNA from enzymatic degradation, transcription termination, export of mRNA from the nucleus and translation.\textsuperscript{28} The addition of the poly(A) tail involves first cleaving the residue at the 3’-end of the pre-mRNA \textit{via} a multiprotein complex.\textsuperscript{28} The poly(A) tail is then built by the addition of adenosine monophosphate residues, by the enzyme polyadenylate polymerase in concert with components from the multiprotein complex. This process continues until the tail is approximately 250 nucleotides long, at which point the tail becomes too long for the polyadenylation to be continued by the bound multiprotein complex and polyadenylation stops.\textsuperscript{28}

1.5 Splicing

Splicing is the final part of RNA processing, where pre-mRNA is converted into mature mRNA \textit{via} the excision of unwanted nucleic acid sequences.\textsuperscript{29} The structure of pre-mRNA consists of exons and introns; both are sequences of nucleotides. Exons are comprised of exact sequences which are then translated into the primary structure of peptides and proteins. Where as, introns are non-coding sequences which need to be removed, to produce the mature mRNA for translation (Figure 5). This intron excision is called ‘splicing’ and is achieved through two transesterification reactions.
In the first transesterification step, the 2’-hydroxyl of an adenosine residue, located at the intron branch point, performs a nucleophillic attack on the phosphodiester bond of a guanosine residue, which directly links the intron to the 5’-exon (Figure 5). This reaction replaces the 5’-, 3’-phosphodiester bond between the intron and the 5’-exon, with an internal 5’-, 3’-phosphodiester bond at the intron branch site, resulting in a “lariat” type intermediate structure. This results in two products, the lariat intron 3’-exon and a separate 5’-exon. However, both of these products are still associated with each other in the intermediate complex, the exact details of which will be outlined in section 1.6.

**Figure 5:** The process of mRNA splicing.

**Figure 6:** Mechanisms for the transesterification reactions during intron splicing.
The second transesterification step occurs when the 3’-hydroxyl of the terminal ribose of the 5’-exons performs a nucleophillic attack on the phosphorodiester group which links the intron to the 3’-exon (Figure 6). This reaction involves breaking the phosphodiester bond which connects the intron lariat to the 3’-exon, forming a phosphodiester bond between the two exons. This results in the lariat intron sequence being excised, leaving only the exon sequences joined contiguously in the mature mRNA.

In some instances, these two chemically simple transesterification steps are known to occur in nature via self-splicing catalysed directly by the intron RNA itself, e.g. the group II introns.\textsuperscript{30,31} However, in eukaryotic cells splicing can only be performed when it is catalysed by a complex macromolecular machine known as the spliceosome. The spliceosome provides a surprising amount of mechanistic complexity to what otherwise might be considered a relatively simple biological process.

1.6 The Spliceosome

The spliceosome is a macromolecular complex with a core structure composed of five small nuclear ribonucleoproteins (snRNPs).\textsuperscript{32-35} These snRNPs (U1, U2, U4, U5 and U6) contain one or more uridine-rich small nuclear RNAs complexed with various proteins. These abundant snRNPs are stored in subnuclear domains, known as speckles, before being recruited into the spliceosome. They are also thought to effectively act as ribozymes,\textsuperscript{36} for the two transesterification reactions of the splicing cycle, as the RNA component of the snRNA is thought to both stabilise the intermediate steps of the process and direct all of the interactions with the pre-mRNA.

The spliceosome is involved in catalysing the majority of eukaryotic gene splicing events. Currently, the characterisation of the spliceosome is at a relatively early stage, and there are many things that are not well understood. The current state of our knowledge of the spliceosome and its function in the splicing cycle is shown in Section 1.6, an explanation of the difficulties associated with the study of the spliceosome is provided in Section 1.7.
1.7 The Splicing Cycle

It was explained in Section 1.2 how the two steps of splicing occur chemically in eukaryotic cells, and that this process is catalysed by the spliceosome, however the exact mode of action for this catalysis is still an active area of research. Currently, the catalytic cycle for splicing has not been fully characterised or completely understood, though there is strong evidence for the mechanism shown in Figure 7.\(^{37}\)

The spliceosome snRNPs effectively act as its ‘building blocks’, with these components assembling and disassembling in a stepwise fashion during splicing. There are also various rearrangement steps involved in this catalytic mechanism, which is termed the splicing cycle.

What is still poorly understood is how the different regions of the intron are recognised by the appropriate ribonucleoproteins within the spliceosome. Recent evidence appears to suggest that the spliceosome is formed via several steps, before splicing occurs (Figure 6). The first of these steps, appears to be the formation of the engagement complex (complex E), wherein the snRNPs U1 and U2 bind to the pre-mRNA. The second step of this cycle is a conformational rearrangement, where U1 and U2 bind one another to form complex A. Subsequent association with tri-snRNP [U5:U4/U6] forms complex B. U5 snRNP binds to the tri-snRNP complex via protein-protein interactions, while U4 and U6 forms a discontinuous RNA helix. The next step after the addition of the tri-snRNP, involves the dissociation of U1 and U4 from complex B, and an association of a separate protein complex known as the Nineteen complex (NTC),\(^{38}\) to form complex C. The NTC is named due to the protein Prp19 acting as the core protein.
The SnRNPs U2 and U6 are known to form a discontinuous helix at the core of the spliceosome, which bind to the intron branch point and the 5'-exon (Figure 8). This interaction allows the spliceosome to correctly position itself to select the reaction site for transesterification during the first stage of splicing.\(^ {39}\)
Figure 8: Schematic of interactions between the U2/U6 snRNP bicomplex and a pre-mRNA intron. Other snRNPs are omitted for clarity.

When the first stage of splicing is completed it produces two products, the free 5′-exon and the intron 3′-exon. These products are still bound to the spliceosome via U5, this snRNP serves to ‘tether’ the two products together and assists in the alignment of the 5′-exon with the 3′-exon intron complex. Therefore, such alignment allows the second transesterification reaction to occur (Figure 9).

Figure 9: Schematic of interactions of the snRNP U5 with the pre-mRNA (other snRNPs are omitted for clarity).

Once this second transesterification reaction has taken place the mature mRNA product dissociates from the intron lariat-spliceosome complex. The spliceosome-intron complex is then disassembled, and its components are recycled for use in further splicing cycles.
Little research has been carried out on this disassembly step and it is still unknown if there is a facilitator for this process, or if it simply ‘falls apart’ naturally after use.

1.8 Structure of the Spliceosome

The spliceosome is one of the most complex macromolecular machines present within eukaryotic cells. It was determined relatively early during the analysis of spliceosome that it consists of snRNPs, and that these snRNPs were integral to the splicing cycle. A more puzzling aspect of the spliceosome is that there are proteins with no apparent association with any of these snRNPs, which are also integral to splicing.

But due to the sheer number of protein and RNA components in the spliceosome,\textsuperscript{39} it has to date, defied conventional methods for conclusive characterisation (x-ray crystallography, etc). Even if these techniques could be employed, they would merely provide a static view of the complex, revealing little of the dynamic nature of the spliceosome. This deficiency has led to a greater interest in the analysis of the spliceosomes individual structural components, and their function in splicing. Advances in protein mass spectrometry and enhanced purification techniques have led to a significant improvement of our understanding of these aspects.

New techniques in the purification of the spliceosome, such as the tobramycin affinity-selection method,\textsuperscript{41} have enabled its analysis. This has indicated with little ambiguity, which proteins are, and are not, directly associated with the spliceosome complex. These techniques, coupled with advances in protein mass spectrometry, have led to the discovery that there are over 300 distinct proteins in the spliceosome, far more than had been previously thought. Interestingly, the vast majority of these proteins are considered splicing factors; \textit{i.e.} there are few, if any, redundant proteins that serve no function in splicing. However, these purification techniques can give rise to physical disruption, which in turn can cause false readings for these proteins. Furthermore, the mass spectrometry provides no information on the stoichiometry of these proteins with respect to the larger structure.
The complexity of the spliceosome is somewhat surprising because the two transesterification steps are relatively simple biochemical reactions. There are examples in nature where splicing occurs in which proteins play no catalytic role. This autocatalytic excision of introns is rare, only occurring for the group I-III introns present in pre-mRNAs of fungal organelles. This self-catalytic pathway performs many of the functions of the spliceosome using the structure and functional groups of the RNA intron alone.\textsuperscript{42}

Several explanations have been offered to account for the complex nature of the spliceosome, the first of which is that splicing of the nucleotides has to be incredibly accurate. A mistake in the position of splicing can result in a nonsense mutation, caused by the frame-shift in the mRNA product. As a result, cells have numerous checking mechanisms\textsuperscript{43} to ensure that the mRNA produced does not contain such defects. These checking mechanisms (termed mRNA surveillance) occur at every major step of the splicing cycle, ensuring correct intron excision occurs and directing defective mRNAs into degradation pathways. When such defective mRNAs are produced they are known to quickly degrade, as such it is possible that many spliceosomal proteins are not involved in splicing itself, but are instead involved in these checking mechanisms.

Another possible reason for the apparent complexity of the spliceosome is that the mRNA produced is associated a multicomponent complex known as the exon-junction complex (EJC).\textsuperscript{44} This complex is positioned approximately 20 nucleotides from the exon-exon ligation point, and is known to have multiple functions in splicing. The EJC identifies inappropriate stop codons produced by errors in splicing, as such it is involved in the recognition of mature mRNA, allowing for subsequent transportation from the nucleus to the cytoplasm. Thus, proteins associated with the EJC, are not involved in catalysing splicing, but are instead involved in post-splicing mRNA surveillance mechanisms.

A final explanation for the splicesomes complexity is that the process of transcription, which precedes splicing, is intimately connected to the mRNA process.\textsuperscript{45} In some cases it is thought that splicing occurs before the synthesis of the pre-mRNA is completed, and
that this temporal overlap plays a role in ensuring the accuracy of splicing. There is thought to be ‘adaptor’ proteins that link the splicing machinery to the RNA synthesis machinery. It is these proteins which could add to the overall apparent complexity of the spliceosome.

1.9 Difficulties Encountered With Spliceosome Studies
As highlighted in chapter 1.4, the spliceosome’s complexity and dynamic nature makes characterising its functions problematic. These problems are very similar to those faced when attempts were first made to understand the ribosome. The ribosome is a complex macromolecular complex, similar to the spliceosome. However, the structure and function of the ribosome has largely been characterised over several decades.46-49

The dynamic nature of the ribosome had proved to be a hindrance to the various analytical and biophysical tools conventionally used to study protein complexes, which benefit from the relatively static nature of most systems. This problem was addressed by exploiting the abundance of antibiotics known to inhibit the various stages of ribosomal translation.50-54 These antibiotics function through binding specific components of the prokaryotic ribosome, therefore blocking any further progress. This effectively ‘stalls’ the process, and allows characterisation studies to be performed on the structure and function of these intermediates, which would otherwise be impossible.

But while there have been ample natural products available to inhibit the ribosome, the same abundance has not been found for other macromolecular complexes, especially those specific to eukaryotic cells, such as the spliceosome. There is, therefore, a pressing demand for inhibitors of the spliceosome, which would allow for the isolation and analysis of complexes formed by the splicing cycle.

1.10 Current Methods of Splicing Inhibition
During splicing, there are five major complexes formed by the spliceosome (Figure 7, page 24), each of these complexes (E, A, B, C, D) performs important functions during
splicing. In addition, there is also a complex designated as H, composed of numerous snRNP proteins and forms on any single stranded RNA regardless of whether splicing is required. This complex appears to form before complex E in the spliceosome pathway.

The task facing research groups in the analysis of the spliceosome is to find ways to ‘stall’ the assembly/disassembly of the spliceosome at each of these stages. So far there has been various methods employed to achieve this, many of these involve the depletion or removal of functionally important elements that the spliceosome needs to continue the splicing pathway (catalytic proteins, binding sites, etc.). As mentioned in Section 1.8, a method used in the analysis of dynamic systems is inhibition via small molecule inhibitors, and this methodology can be applied to the spliceosome.

A number of methods have been found to successfully isolate the various spliceosomal complexes produced during splicing. However, all these methods involve indirect splicing inhibition. All of these methods have in some way changed the spliceosome or have sequestered some component which is integral for splicing. The spliceosomal complexes H, E, A, B, and C can be identified and resolved in native gels, so analyses of these methods of spliceosome inhibition are possible.\textsuperscript{55,56}

Complex E can be accumulated via the depletion of the U2 snRNA, which is achieved by RNase H-mediated digestion. This involves using a specific complementary DNA oligonucleotide to bind to bind to the U2 snRNA, which causes native RNase H to cleave the U2 snRNP.\textsuperscript{31} Another way of accumulating complex E is via the use of a 2’-O-methylated analogue of oligonucleotide U2b, which specifically binds to the branch point of the intron. This addition blocks the recruitment of U2 snRNP into the spliceosome, preventing the formation of complex A.\textsuperscript{57,58}

Complex A can be accumulated via two methods, which are similar to those employed in the accumulation of complex E. The first method involves the specific depletion any of the three snRNPs (U4-U6) via the use of RNase H-mediated digestion with an antisense DNA oligonucleotide.\textsuperscript{59,60} The second method is to again use a 2’-O-methylated
oligonucleotide to interfere with either the base pairings essential for the either U4/U6 or U2/U6 complex formation. This inhibition prevents the addition of the tri-snRNP and the formation of complex B.\textsuperscript{61-63}

Accumulating complex B has proved to be more difficult due to it naturally eliminating the U1 and U4 snRNPs to form complex C. Therefore, there are no clear targets which can be blocked or depleted to prevent complex C formation. To date, most studies have made use of the relatively low rate of spliceosome assembly \textit{in vitro}. Ten to thirty minutes after the initiation of splicing of pre-mRNA, mammalian extracts contain primarily complexes A and B, and splicing reactions can then be arrested by simply putting them on ice.\textsuperscript{64,65} A second method for complex B accumulation involves the depletion of the 15.5 kDa protein.\textsuperscript{66} This protein belongs to the [U5:U4/U6] tri-snRNP complex, but has not yet been assigned to a specific snRNP. It is known that the 15.5 kDa protein is essential for the unwinding of the U4/U6 snRNPs allowing for the dissociation of U4. This unwinding function is connected to its binding to the 5'-stem-loop of U4 snRNP. The removal of this protein therefore leads to the accumulation of complex B, as U4 dissociation is integral to the formation of complex C.

Complex C has been accumulated \textit{via} the conversion of the phosphodiester group at the 5'-splice site, into an $R_P$-phosphorothioate\textsuperscript{67} (Figure 10). Phosphorothioates are phosphate analogues in which one of the non-bridging oxygen atoms is replaced with sulfur ($R_P$ refers to the chirality of the phosphorothioate). The sulfur atom then disrupts subsequent chemistry\textsuperscript{68} preventing the formation of the 5', 2'-phosphodiester bond and therefore, the formation of complex C.

A second way of preventing the first \textit{trans}-esterification reaction is to mutate the branch point sequence. The TACTAAC intron sequence of the pre-mRNA was mutated to TACTACC\textsuperscript{69}, which eliminated the intron branch point. This leads to the branch point not being recognised by the spliceosome, and this sequence is not positioned correctly to carry out the nucleophilic attack of the phosphodiester bond at the splice site, leading to the accumulation of complex C.
Complex D was also accumulated via a modification of this method, with the 3'-splice site being targeted for conversion to the $R_P$-phosphorothioate.

Another successful approach employed a series of antisense 2’-O-allyl oligonucleotides, complementary to U2 snRNA, in an attempt to block its function in the second transesterification step. The most successful of the 2’-O-allyl oligonucleotides investigated was complementary to nucleotides 57-68 of the U2 snSNA, which corresponds to the stem-loop IIa region of this snRNA.

The isolation of these splicing complexes has led to our current knowledge of the mechanism and composition of the spliceosome. New ways of inhibiting splicing and the spliceosome, preferably at intermediate stages between the known complexes, should hopefully lead to an even greater understanding of the splicing cycle.

1.11 Small Molecules Splicing Inhibitors
Comparatively few small molecule compounds have been found which inhibits splicing. Those that have been found, appear to lead to the accumulation of the earlier forms of the spliceosome, e.g. complexes E, A and B.
<table>
<thead>
<tr>
<th>Complex</th>
<th>Small molecule inhibitor</th>
<th>Strategy</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>Kanamycin 1, Streptomycin 2 and Neomycin 3</td>
<td>Proposed inhibition via Direct interaction with the snRNA components of the spliceosome complexes. See Section 1.11.2.</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>Oxspiro 4</td>
<td>Interactions with protein components of the spliceosome present during splicing. See Section 1.11.2.</td>
<td>6</td>
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<tr>
<td>E</td>
<td>Disopyrin 5</td>
<td>Targeting splicing factor SF2/ASF via known topoisomerase I inhibitors. See Section 1.11.3.</td>
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<tr>
<td>E</td>
<td>Pladienolide B 25</td>
<td>Binding to the SF3b protein complex in U2 snRNP. See Section 1.11.5.</td>
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</tr>
<tr>
<td>E</td>
<td>Spliceostatin A 26</td>
<td>Binding to the SF3b protein complex in U2 snRNP. See Section 1.11.5.</td>
<td>4,73</td>
</tr>
<tr>
<td>A</td>
<td>Anacardic acid 6 and Garcinol 7</td>
<td>Blocking Pre-mRNA splicing before 1st catalytic step via protein acetylation inhibitors. See Section 1.11.1.</td>
<td>5</td>
</tr>
<tr>
<td>A &amp; B</td>
<td>Oxspiro 8</td>
<td>Interactions with protein components of the spliceosome present during splicing. See Section 1.11.2.</td>
<td>6</td>
</tr>
<tr>
<td>B</td>
<td>Butyrolactone 3 9</td>
<td>Blocking Pre-mRNA splicing before 1st catalytic step via protein acetylation inhibitors. See Section 1.11.1.</td>
<td>5</td>
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<td>B</td>
<td>Oxspiro 10</td>
<td>Interactions with protein components of the spliceosome present during splicing. See Section 1.11.2.</td>
<td>6</td>
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<tr>
<td>B</td>
<td>Suberoylanilide hydroxamic acid 11, Splitomicin 12 and Dihydrocoumarin 13</td>
<td>Blocking Pre-mRNA splicing before 1st catalytic step via protein deacetylases. See Section 1.11.1.</td>
<td>5</td>
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<tr>
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<td>Disopyrin 14</td>
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<tr>
<td>B</td>
<td>Disopyrin 15 and 16</td>
<td>Targeting splicing factor SF2/ASF via known topoisomerase I inhibitors. See Section 1.11.3.</td>
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<tr>
<td>D</td>
<td>Indole derivative 17</td>
<td>SR proteins of protein splicing factor SF2/ASF prevalent in HIV-1 pre-mRNA. See Section 1.11.6</td>
<td>74</td>
</tr>
</tbody>
</table>

Table 1: Small molecule inhibitors of the Spliceosome
One reason for this trend could be that once complex B is formed, all snRNPs are bound to the pre-mRNA. Therefore, in order to accumulate later splicing complexes, small molecule inhibitors would have to block a function that is not required for spliceosome assembly, but is essential to the transesterification reaction steps.

So far the most commonly employed method for finding these small molecule inhibitors appears to be identifying a specific target, such as a specific interaction or catalytic step. This then provides a starting point in the search for an inhibitor.

There is however a difference between indirect splicing inhibitors and spliceosome binders. Indirect splicing inhibitor modifies a part of the spliceosome in some way, thereby disrupting its associated function. This inhibits the splicing indirectly (similar to the methods detailed in Section 1.6) but using a small molecule to achieve this. The alternative is direct binding to the spliceosome with a small molecule (usually via interaction with a spliceosomal protein). Although finding small molecule inhibitors that actually bind to parts of the spliceosome is far more difficult, they do have the advantage of being useful for static analytical techniques, such as X-ray structural analysis. It could also lead to novel drugs for diseases associated with incorrect splicing (e.g. spinal muscular atrophy, certain types of cancer).
1.11.1 Splicing Inhibition via the use of Histone Deacetylase and Histone Acetyltransferase Inhibitors

It is known through the purification of mixed populations of spliceosomal complexes that histone deacetylases (HDACs) are associated with the spliceosome.\textsuperscript{75-77} HDACs catalyse the deacetylation of the ε-amino group of activated acetylated lysine residues in a multitude of proteins (Figure 11),\textsuperscript{5} and it has been suspected that this process plays some part in the regulation of splicing.\textsuperscript{78,79}

![Diagram of Histone Modification](image)

**Figure 11**: Histone deacetylases (HDAC) and histone acetyltransferases (HATs) modify snRNPs in the spliceosome.

These HDACs provided a splicing inhibition target with already well known inhibitors, and was explored by Khun et al.\textsuperscript{80} Zn$^{2+}$ dependent histone deacetylases (known as HDAC classes I and II), such as suberoylanilide hydroxamic acid 11 (SAHA) were used as inhibitors. These deacetylases were found to block splicing before its 1$^{st}$ catalytic step.

Suberoyl bis-hydroxamic acid 18 (SBHA) and Suberoylanilide 19 (SA) have similar functionality to SAHA 11, but are not HDAC inhibitors. These compounds were used to test whether HDAC inhibition plays a major role in the splicing inhibition activity observed from SAHA, or whether another mode of action is involved. Results thus far indicate this to be the case as, unlike SAHA, both SBHA and SA showed no splicing inhibition activity. The class III histone deacetylases, which use a different mechanism...
for deacetylation\textsuperscript{81,82} were also targeted with the specific inhibitors splitomicin and dihydrocoumarin. These compounds also showed inhibitory effects, blocking the first catalytic step of splicing.

Histone acetyltransferases (HATs) are enzymes capable of catalysing the acetylation of proteins, and were identified as being present in mixed populations spliceosomes. HATs also have a number of identified inhibitors, and three of these (anacardic acid \textsuperscript{6}, garcinol \textsuperscript{7} and butyrolactone \textsuperscript{3 9}) were chosen as splicing inhibitor candidates. All three were observed to inhibit pre-mRNA splicing.

In common with protein deacetylases, the protein acetylation inhibitors arrested splicing at its first catalytic step, with anacardic acid and garcinol showing slightly higher levels of inhibitory activity than butyrolactone \textsuperscript{3 9}.

Blocking the splicing activity before the first step of catalysis does not mean that these compounds all inhibit spliceosome at the same stage. Complexes E, A, B and C are all formed before the first transesterification reaction takes place. The inhibitors butyrolactone \textsuperscript{3 9}, SAHA \textsuperscript{11}, splitomicin \textsuperscript{12}, and dihydrocoumarin \textsuperscript{13} all resulted in an increase in the accumulation of complex B, whilst anacardic acid \textsuperscript{6} lead to the accumulation of complex A (Figure 12). Garcinol \textsuperscript{7} lead to accumulation of small amounts of complex A, although this appeared to decrease as the experiment proceeded. This implies that garcinol \textsuperscript{7} actually destabilises spliceosome complex B entirely.
Figure 12: The stages of spliceosome complex inhibition observed for various HDAC and HAT inhibitors.

The protein compositions of both the A and B complexes were analysed. However, these small molecule splicing inhibitors are an example of indirect splicing inhibition. These molecules either deacetylate or block acetylation of various protein components involved with the spliceosome. This, in turn inhibits the function of the spliceosome in some fashion, causing either complex A or B to be accumulation.

1.11.2 Splicing Inhibition via Broad Range Antibiotics

The use of broad-spectrum antibiotics as inhibitors of ribosomal function is a well-used investigative tool, their possible use as splicing inhibitors was explored by Aukema et al. The Aukema group compared inhibition traits between human splicing and splicing in Saccharomyces cerevisiae, which at that time had no reported splicing inhibitors. Over 26 different antibiotics were tested, together displaying a wide-range of biological activities (antibacterial, insecticidal, anti-inflammatory, etc). It was found that the most potent of these compounds belonged to the aminoglycoside and oxaspiro families of antibiotics.
The aminoglycosides have historically been used to treat a variety of bacterial infections, including tuberculosis. These compounds are known as ribosome and ribozyme inhibitors, which work via direct interaction with RNA. From this family of antibiotics, kanamycin, streptomycin and neomycin were tested and found to have significant splicing inhibition activity (Table 2). The Aukema group’s initial hypothesis was that this inhibitory activity stems from a similar mode of action to their ribosomal inhibition activity, i.e. through direct interaction with the snRNA components of the spliceosome complexes.

The oxaspiro compounds are thought to be potential intermediates in the biosynthesis of manumycin A 20, a farnesyltransferase inhibitor and a known antitumor agent. From the oxaspiro family, compounds 4, 8 and 10 were tested and exhibited similar low IC\textsubscript{50} values to the aminoglycosides. Since the oxaspiro compounds are intermediates in the biosynthesis of a known enzyme inhibitor, it was hypothesised that the splicing inhibition activity shown by these compounds was due to interactions with protein components of the spliceosome present during splicing.
Denaturing gel analysis was also performed on these inhibited splicing reactions to ascertain which splicing complex accumulated. The scope of this technique is somewhat limited, as the only complexes that can be observed via this technique are spliceosomal complexes A and B. When compounds 1, 2, 3 and 4 were tested, there was no accumulation of either spliceosomal complex A or B and no spliced mRNA product was observed. This result implies that these compounds inhibit splicing before either of these complexes form. However, there was an observed accumulation of spliceosomal complexes when the related oxaspiro compounds 8 and 10 were used. When oxaspiro 8 was tested, both A and B complexes were formed in roughly equal amounts. Whilst, when oxaspiro 10 was tested, complex A was only present in minimal amounts. Both of these oxaspiro compounds prevent mRNA splicing and the levels of spliceosomal complexes formed remained stable, regardless of how much time was allowed to pass. This result implies that oxaspiro 10 inhibits the splicing cycle strongly after the formation of complex B, but has no inhibitory effect upon earlier steps of the cycle. Oxaspiro 8 appears to have a more subtle effect, inhibiting the splicing cycle at steps both before and after the formation of complex B. Due to the similarities in the nature of the observed inhibition for compounds 8 and 10, it is reasonable to assume that the inhibitory effect that occurs after the formation of complex B is the same. It is interesting to note that the

<table>
<thead>
<tr>
<th>Splicing Inhibitor</th>
<th>IC₅₀ (mM)</th>
<th>Spliceosome complex accumulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin 1</td>
<td>0.4 +/- 0.3</td>
<td>None detected</td>
</tr>
<tr>
<td>Streptomycin 2</td>
<td>1.0 +/- 0.5</td>
<td>None detected</td>
</tr>
<tr>
<td>Neomycin 3</td>
<td>0.08 +/- 0.02</td>
<td>None detected</td>
</tr>
<tr>
<td>Oxaspiro 4</td>
<td>0.7 +/- 0.1</td>
<td>None detected</td>
</tr>
<tr>
<td>Oxaspiro 8</td>
<td>0.8 +/- 0.2</td>
<td>A, B</td>
</tr>
<tr>
<td>Oxaspiro 10</td>
<td>0.6 +/- 0.2</td>
<td>B, minor A</td>
</tr>
</tbody>
</table>

Table 2: Levels of splicing inhibition and spliceosomal complexes observed for various potential splicing inhibitors tested in Saccharomyces cerevisiae.⁶
inhibitory effect which occurs before complex B formation appears to be greatly diminished by the replacement of the acetamide group in oxaspiro 8, with a benzamide group in compound 10. As yet no explanation for this effect has been put forward, and this observation deserves further investigation.

1.11.3 Splicing Inhibition via Diospyrin Derivatives

Serine rich proteins (SR proteins) have been implicated in the regulation and selection of splice sites in eukaryotic pre-mRNA. SR proteins are also known to be targets for the kinase activity of topoisomerase I, which phosphorylates arginine and serine rich domains. Topoisomerase I is better known as an enzyme which cuts one strand of the DNA duplex then reanneals the cut strand, preventing overwinding of the DNA helix. The importance of this enzyme has led to the discovery of a number of inhibitors which target the DNA-topoisomerase I complex. In contrast, the kinase activity of topoisomerase I has received far less attention, although there have been inhibitors reported from the diospyrin family of compounds. Diospyrin itself and isodiospyrin have been shown to inhibit topoisomerase I from Leishmania donovani and humans respectively. Since topoisomerase I is present during splicing, diospyrin 16 provided a starting point for the search for small molecule splicing inhibitors, as investigated by Tazi et al. Using this inhibitor the Tazi group targeted the SR protein, and known splicing factor, SF2/ASF. SF2/ASF has arginine and serine rich domains making it an ideal target.

![Diospyrin derivatives](image)

Of the diospyrin derivatives that were tested, compounds 5, 14, 15 and 16 (stereochemistry undefined in original paper) showed inhibition of mature mRNA
formation. Interestingly, whilst compounds **5** and **14** led to the accumulation of pre-mRNA, **15** and **16** led to the accumulation of splicing intermediates, *i.e.* the lariat intron 3′-exon and free 5′-exon.

**Figure 13:** Inhibition of splicing by various diospyrin derivatives occurs at different stages of the splicing cycle.

The extent of pre-mRNA splicing was tested with native-gel electrophoresis following incubation with various diospyrin derivatives which, when performed with unobstructed pre-mRNA splicing, can be used to monitor the formation and dissociation of spliceosomal complexes A and B. When the diospyrin compounds **15** and **16** were tested, no accumulation of complex A or B was observed. This result was expected, due to previous results implying that compounds **15** and **16** inhibit splicing after the first splicing reaction has taken place. There was an accumulation of complex B for compound **14**, but no accumulation of either complex A or B for compound **5**, implying that compound **5** inhibits earlier in the cycle than compound **14** (Figure 13). The modification of functionality on the bis-naphthoquinoid core appears to influence which step of splicing is inhibited, this interesting effect appears to be unique among splicing inhibitors.
1.11.4 Splicing Inhibitor Searches via High-Throughput Screening

The use of high-throughput screening with compound libraries is an underutilised technique as a method of discovering splicing inhibitors. To date, the only published data was presented by O’Brien et al. The O’Brien group tested over 8000 commercially available natural products and synthetic compounds were tested, producing a vast amount of splicing inhibition data.

This data was converted into statistical Z-scores to allow for easier comparison. The vast majority of compounds tested had Z-scores equivalent to control DMSO treatments (Z ≤ 2.5), with a few reaching slightly higher to Z = 4 or 5 indicating only moderate splicing inhibition. However, the antitumor agent isoginkgetin was found to be the strongest splicing inhibitor tested with a Z-score of Z = 27.9. Native-gel electrophoresis was also performed, which revealed that the presence of isoginkgetin led to the accumulation of spliceosomal complex A.

1.11.5 Inhibition of HIV Splicing Factor SF2/ASF

Current treatments for HIV-1 infection involve targeting the viral proteins, reverse transcriptase, protease and gp120. However, HIV-1 has been found to acquire resistance to all of these targets, and transmission of these drug resistant strains is common. A potential new method for treatment of HIV-1 is via targeting HIV-1 mRNA splicing. HIV-1 gene expression in some instances involves alternative splicing, which is where exons of pre-mRNA can be combined in several different arrangements during splicing. This produces multiple mRNA products from a single gene that are then translated into different protein isoforms. HIV-1 splicing involves SR proteins, which are non-spliceosomal nuclear RNA-binding proteins, which interact with HIV pre-mRNA and are
thought to assist in the alternative splicing of over forty different mRNAs from the single full length HIV-1 pre-mRNA transcript. One of these SR proteins, called SF2/ASF, appears to play a major role in this regulation of pre-mRNA splicing during HIV-1 infection, and was targeted for small molecule inhibition by Bakkour et al. 

The Bakkour group screened a large collection of compounds, and discovered that several benzopyridoindole and pyridicarbazole derivatives inhibited the SF2/ASF protein. Of the 220 indole derivatives tested in the study, compound 17 was found to have the highest activity, and efficiently blocked HIV-1 viral production in infected macrophages and in clinical isolates from infected patients.

While this method lends itself to drug discovery, it is of limited use in spliceosome analysis. Indole derivative 17 blocked splicing at the earliest stage, effectively preventing the spliceosome from forming.

1.11.6 Inhibition of Protein Complex SF3b

Splicing factor SF3b is a multi-protein subcomplex of the U2 snRNP, and is involved in binding of this snRNP to the branch point of pre-mRNA. This protein is known to be essential for pre-mRNA splicing, and therefore represents a viable target for splicing inhibition. Both spliceostatin A 26 and pladienolide B 25 were found to bind to proteins in this complex. Pladienolides a family of naturally occurring compounds which exhibit antitumor activity. This antitumor activity may or may not be connected to splicing activity exhibited by pladienolide B 25.
Spliceostatin A 26 is the methylated derivative of the secondary metabolite FR901464 27, which was isolated from a culture of the bacterium *Pseudomonas* sp. 2663. FR901464 27 is historically known as an anticancer agent, which has strong activity against the polyomavirus, Simian virus 40 (SV40). It was also known that FR901464 27 reduces mature mRNA levels, but the mode of action was unclear until it was discovered that Spliceostatin A 26 binds to protein complex SF3b. There is a published total synthesis of FR901464 27, which will aid in the synthesis and study of further derivatives in the event of any therapeutic benefits being reported.

Of all the compounds tested, spliceostatin derivative 28 showed the greatest activity. All three compounds led to the accumulation of unspliced mRNA, and blocked splicing after the initial step of spliceosome assembly, leading to the accumulation of complex E. These results seem to consolidate the theory that SF3b regulates the binding of the U2 snRNP to the branch point in the intron. Interestingly, the unspliced mRNA was still capable of being exported and translated, despite still containing introns.
Although the biotinylated spliceostatin A derivative 29 had a greatly reduced activity, this modification allows for detection of protein binding partners by SDS-PAGE, Coomassie staining and LC-MS/MS analysis. These techniques showed that spliceostatin A binds to proteins SAP155, SAP145 and SAP130, which are known to be a part of the SF3b protein subcomplex. Modifications have been performed previously on the core structure of spliceostatin A to try to increase the antitumor activity and an analysis of structure-activity relationship is shown below (Figure 14).

![Structure diagram]

Figure 14: FR901464 27 structure-activity relationship analysis via testing of derivatives.

The epoxide moiety appears to be essential for splicing inhibition, as replacement of the oxygen with a carbon atom effectively eliminated the observed inhibitory activity. When the stereochemistry of the epoxide was inverted, this also led to a decrease in the observed inhibitory activity. The C-2 hydroxyl appear to have a negative role in splicing inhibition activity, as alkylation actually increases inhibition, with the best result being when a methyl group is used. The C-5 hydroxyl seems to play an important role in splicing inhibition, as when group was acetylated the levels of mature mRNA was equivalent to control samples. The side chain removal and modifications have to date not increased its activity, but many of these derivatives have equivalent or slightly reduced activity. This implies the side chain may have a minor role in binding, but not an essential one.
1.11.7 Summary of Small Molecule Inhibitors

The targeting of enzymes involved in the regulation of pre-mRNA splicing for small molecule inhibition, was explored by both Hildmann and Tazi et al,\textsuperscript{71,117} and are excellent examples of indirect splicing inhibition. The inhibition of these enzymes prevents an essential function in the splicing cycle being carried out, pausing the spliceosome at various points in the splicing cycle. However, they do not directly bind to any part of the spliceosome. The exact modes of action of some of the splicing inhibitors mentioned earlier are still unknown. This highlights a major problem with finding splicing inhibitors, it is very difficult to identify binding targets among the hundreds of proteins present in the spliceosome. This is why techniques such as high-throughput screening of compound libraries\textsuperscript{118} is of relatively limited use, for example over 8000 compounds were tested by O’Brien et al\textsuperscript{7} with only one highly effective splicing inhibitor found and its mode of action remains unknown. These examples highlight the need to prioritise targets within the spliceosome, if there is any hope of determining the exact nature of inhibitors found.

The SR protein SF2/ASF was investigated by the Bakkour group, as a target for small molecule inhibitors of HIV-1 reproduction. SR proteins are present during splicing, but are not associated with the spliceosome directly, making them significantly easier to isolate. This allowed for a much more precise analysis of the mode of action for the indole inhibitors identified. To date, only two examples of true spliceosome inhibitors have been found spliceostatin A and pladienolide B. Furthermore, there is information on the exact position in the spliceosome where these molecules exert their effect. Spliceostatin A is the only inhibitor for which any attempt has been made to optimise the efficiency of spliceosome binding. So while there has been progress made,\textsuperscript{119} the search for small molecule inhibitors of spliceosome function is still in its infancy. The splicing cycle is still far from fully characterised, and the search for these small molecules that can inhibit splicing, and provide further mechanistic insights, continues.
2.0 Overall Objectives of this Project

- To select a suitable target for splicing inhibition, review previous work on this target, and identify any leads into potential small molecule splicing inhibitors.

- To research these leads and compose a list of small molecules that could have splicing inhibition activity against our selected target. This set of small molecules could then be streamlined depending on availability/ease of synthesis and likelihood of activity.

- To test these potential inhibitors using standard splicing inhibition assays, allowing for the evaluation splicing inhibition activity.

- To analyse the structure-activity relationship for the most active small molecule inhibitors.

- To synthesise a directed library of derivatives based on the core structure of this inhibitor. These derivatives will hopefully improve on the base value of splicing inhibitory activity shown by the parental compound.

If these goals are successful, this project should provide key information on the inhibition target and facilitate the analysis of the spliceosome as a whole. This will hopefully provide a valuable tool in the on-going characterisation of the spliceosome, and may even provide the basis for the rational design of future drugs to combat diseases associated with mRNA splicing.
3.0 Protein hSnu114/Snu114 as a Small Molecule Splicing Target
The proteins present in snRNPs are split into two categories, common proteins that are present in most snRNPs, and particle-specific proteins which are unique to one of the snRNPs. The target chosen in this study for small molecule inhibition, was one of these particle-specific proteins, Snu114.

Snu114 belongs to the U5 snRNP complex and was first reported in 1997 by Fabrizio et al. This protein was chosen as a splicing inhibition target for several reasons, the first and most important being that Snu114 is essential for proper functioning of the spliceosome. More specifically, Snu114 binds GTP and is thought to be a GTPase. Mutations to the GTP binding site of Snu114 led to cell death. This observation implies that Snu114 inhibition inhibits the spliceosome at a critical step in the splicing cycle, and therefore this could allow for investigation and analysis of this critical step. Secondly, as part of the U5 complex, Snu114 is incorporated into the spliceosome after the initial U1/U2 attachment and rearrangement (complex A). It is then involved throughout the two transesterification splicing reactions before detaching with the intron as part of the post-splicing complex. This provides a broad scope of splicing complexes that could potentially be ‘stalled’ and characterised via small molecule splicing inhibition. If there is more than one site available for inhibition of Snu114, then different small molecule inhibitors could lead to the accumulation of different complexes.

The target protein Snu114 also has the advantage of being homologous with a protein present in Saccharomyces cerevisiae (yeast cells). The yeast Snu114 is more available and easier to work with than its human homologue hSnu114, therefore this homology can be exploited in analysis of potential splicing inhibitors.

Overall, these factors provide a compelling case for the investigation of the U5 protein Snu114 as a target for small molecule splicing inhibition.
3.1 The Role of Snu114 in Splicing and the Spliceosome

Although Snu114 has not been fully characterised, there has been significant progress made towards understanding the role of this protein during the splicing cycle (Figure 15).

Figure 15: The role of Snu114 within the U5 snRNP and the overall spliceosome.8

Snu114 forms a stable tri-complex with proteins Prp8 and Brr2, which are also part of the U5 snRNP,121 and this tri-complex has actually been found to form in isolation from the
other U5 snRNP components. It is this tri-protein complex which is responsible for the unwinding of the U4/U6 di-snRNP complex and expulsion of U4. This complex is also involved in the unwinding of the U2/U6 dicomplex and expulsion of U2, which then allows for the reassociation of the U4/U6 dicomplex.\textsuperscript{122}

The Snu114 protein exists in two states, with either GTP- or GDP-bound in the active site. These changes of state are a consequence of its own GTPase activity. Snu114 has two major roles as a component of U5 snRNP, the first being that it is required for the formation of complex B (Figure 15, Box 1). The tri-snRNP complex first associates to the spliceosome, after which the GTP state of Snu114 appears to activate Brr2, causing the unwinding of the U4/U6 snRNP duplex and releasing the U4 snRNP from the complex. In the overall splicing cycle this process occurs as part of the step in which complex B is converted to complex C (Figure 7). The snRNP U1 is also removed during this step, but Snu114 appears to play no role in that event.

The second role Snu114 plays is when GTP state of Snu114 is reformed in the post-splicing complex (Figure 15, Box 2). This GTP state activates Brr2 a second time, unwinding the U2/U6 dicomplex and releasing the U2 snRNP.\textsuperscript{123} This release then allows for the reintroduction of the U4 snRNP and the reformation of the U5, U4-U6 tri-snRNP complex, thus completing the catalytic cycle.

In summary, this entire process appears to be controlled by the GDP/GTP hydrolysis which is catalysed by Snu114, and acts as a ‘trigger’ for Brr2. When GDP is bound to Snu114 the functions of Brr2 are suppressed, whilst when GTP binding activates Brr2 activated.\textsuperscript{123}

Although significant advances in the understanding of Snu114 have been made, there are still aspects of its mode of action that are not fully understood. The regulation of the interchange between the GTP/GDP states of Snu114 is obviously important, but it is still not known exactly how this is achieved.\textsuperscript{124} Snu114 was found to be a substrate for protein phosphatases 1 and 2A, and it is believed that this may facilitate structural
rearrangements between the two splicing reactions. The exact nature and importance of the role played by protein phosphatases is not well understood, and it is unclear whether these enzymes perform functions at other splicing stages.

3.2 The Search for Small Molecule Inhibitors of Snu114

Once Snu114 had been chosen as a target, potential small molecule inhibitors needed to be identified. The search is hampered by the lack of a three dimensional structure from either x-ray crystalgraphic or nmr spectroscopic studies. Hence, the strategy we used to find these inhibitors involved using the Snu114 amino acid sequence in initial BLAST database search, to look for homology with other known proteins. These homologues could provide insight into the mode of action and function of Snu114, and could also provide clues to possible small molecule inhibitors, especially if there are already known inhibitors for these homologues.

3.3 Snu114 Homology with EF-2 and EF-G

Initial Blast searches found two possible homologues of Snu114, the elongation factors EF-2 and EF-G (Figures 16 and 17, Pages 53 and 54). These proteins perform a vital function in the elongation step of translation, the translocation of the ribosome in single-codon steps along the mRNA through the hydrolysis of GTP. EF-2 performs this function in eukaryotes, whilst EF-G is its prokaryotic counterpart. The homology between the amino acid sequences of Snu114 and these elongation factors was analysed, with the aim of finding localised structural similarities between EF-2, EF-G and Snu114.

The structure of Snu114 is comprised of five domains with an acidic N-terminus. The nomenclature of these domains is as follows: four domains are numbered II-V inclusively, with the fifth being the G-domain (boxed, Figure 16 and 17). The G-domain is named as such because this region is where GTP is bound and hydrolysed. As detailed in Section 3.1, the GTP-/GDP-bound states appear to be functionally integral to Snu114. This observation appears to make the G-domain the most promising target within Snu114 for splicing inhibition. The conservation of the G-domain between EF-2 and Snu114 is analysed in depth in Section 3.5, first the homology of the other domains are summerised.
Domains III, IV and V were analysed and found to be highly conserved between Snu114 and the two elongation factors. At the amino acid level the Snu114 domain III was found to have 45% identity, and 66% similarity with EF-2 (i.e. an additional 21% of the amino acids have similar side chains e.g. valine and leucine), while domains IV and V had a combined 50% identity and 70% similarity. Domain II is less well conserved with only 30% identity and 50% similarity. The EF-2, G-domain homology is compared in detail in Section 3.5, but had an overall 65% identity and 75% identity. The elongation factors have no acidic N-terminus, so this region cannot be compared. If the acidic N-terminus plays a role in determining the conformation of Snu114, this could have a negative effect on the three-dimensional structural homology with EF-2 and EF-G.

Overall, the analysis of the amino acid sequence of elongation factor EF-2, implies that it is a close homologue for hSnu114. The strong conservation of the G-domain appears to be a clear indication that EF-2 and Snu114 work in a similar way, when it comes to GTP binding and hydrolysis. Of the two elongation factors studied, the eukaryotic protein EF-2 is appears to be a better homologue for Snu114 than the prokaryotic EF-G protein, with a higher overall identity and similarity.
Figure 16: Amino acid sequence comparison in human Snu114, yeast Snu114 and the eukaryotic elongation factor EF-B. The GB-domain is boxed. (Dark blue = complete homology, light blue = similarity, white = no homology).
Figure 17: Amino acid sequence comparison in human Snu114, yeast Snu114 and the prokaryotic elongation factor EF-G translocase.\textsuperscript{54} The G-domain is boxed. (Dark blue = complete homology, light blue = similarity, white = no homology).
3.4 Snu114 Inhibition via EF-2 Inhibitors

Since EF-2 is considered the closer homologue to Snu114, it was used as a template for our initial work. To date, there has only been one published inhibitor of EF-2, the antifungal secondary metabolite known as sordarin isolated from *Sordaria araneosa*.\(^{126}\)

Sordarin comprises a complex tetracyclic core structure normally referred to as sordaricin, covalently bonded to a C-glycoside, 4-\(O\)-methyl-6-deoxy-\(D\)-altrose (trivially called \(D\)-sordarose). In addition, there is a published crystal structure for EF-2 (Figure 18), which was obtained *via* binding with sordarin.\(^{127}\)

![Sordarin and Sordaricin Structures](image)

This homology between EF-2 and Snu114 suggests that sordarin and sordarin derivatives could be used as splicing inhibitors as well as antifungals. The next step was to conduct an in depth analysis of the amino acid contacts between sordarin and EF-2, and to determine the level of conservation of these residues within Snu114. This will provide information on the likelihood that sordarin will bind to Snu114, and may provide information into possible modifications of sordarin that could improve its affinity for Snu114.

**Figure 18:** Sordarin (grey) bound within EF-2 as determined by the crystal structure by Soe *et al.*\(^{127}\)
3.5 Conservation of Sordarin-EF-2 Amino Acid Contacts in Snu114

As previously mentioned, EF-2 is a remarkable homologue of Snu114, with the G-domain being especially well conserved. A more in depth comparison was made between Snu114 and EF-2, looking for conservation in Snu114 of the amino acids which are responsible for EF-2 binding to sordarin (Figure 19). Six of the nine amino acid contacts (Figure 19; 524, 527, 559, 560, 778 and 801) are completely conserved within hSnu114, while in yeast Snu114 only four sites are completely conserved (Figure 19; 521, 524, 527, and 801), although at residue number 559, valine is conservatively changed to leucine.

![Figure 19](image.png)

Figure 19: Comparison of the G domains of elongation factor EF-2, with yeast Snu114 and hSnu114. Also shown is the amino acid contacts between EF-2 and sordarin (Dark blue = complete homology, light blue = similarity, white = no homology).

The positions of all of the conserved binding sites in both Snu114 and hSnu114 are associated with the tetracyclic core of the sordarin structure in the EF-2 crystal structure (Figure 20). The two residues at 519 and 521, which associate with D-sordarose moiety, are not conserved between EF-2 and either Snu114 or hSnu114. The leucine at 519 is conservatively changed to the non-polar residue isoleucine in Snu114, but in hSnu114 it is drastically changed to the polar residue threonine. The tyrosine present at 521 in EF-2 is even less well conserved, as the equivalent positions are occupied by the basic residue lysine in both Snu114 and hSnu114. These provide an indication that although the key residues which bind to the tetracyclic core appear to be well conserved in Snu114, D-sordarose may disrupt the binding to Snu114.
Figure 20: Sordarin (yellow) within the binding site of EF-2, with key amino acid contacts highlighted (green).
4.0 Sordarin
The overall conservation of binding sites implies that sordarin could inhibit Snu114 in a similar fashion to EF-2. The first task was to investigate sordarin as an EF-2 binder and analyse previous work performed with a view to improving upon the antifungal activity of sordarin.

4.1 The Antifungal Activity of Sordarin
EF-2 is one of three soluble elongation factors which catalyse the elongation phase of translation in fungi. Factors EF-1α and EF-2 are present in all eukaryotic cells but EF-3 is unique and integral to protein synthesis in fungi. Sordarin works as an antifungal by actually binding to the \textit{S. cerevisiae} [EF-2:ribosome] complex and prevents protein synthesis by blocking the release of EF-2 from the post-translocational ribosome.

Sordarin is a secondary metabolite produced by \textit{Sordaria araneosa} and was initially isolated in 1971 by Hauser \textit{et al.} While sordarin was always known to be an antifungal, the mechanism of selective inhibition via binding to the elongation factor [EF-2-ribosome] complex was not discovered until 1997, by Soe \textit{et al.} What is interesting about the mechanism of antifungal activity is that it is unique to sordarin. Other antifungals such as amphotericin B, 5-fluorocytosine and fluconazole target the integrity of the cell membrane through binding of ergosterol or inhibition of its biosynthesis.
This novel aspect of sordarin has resulted in a number of studies being performed focusing on improving its antifungal potency through various synthetic modifications.\textsuperscript{133,134} As a general rule these methods involve the biosynthesis of sordarin based on the 1971 method and removal of the D-sordarose ring by acid catalysed hydrolysis to produce the aglycone, sordaricin 32. Once sordaricin has been produced the new hydroxyl group is used in a wide variety of reactions to produce a directed library of compounds.

\section*{4.2 The Biosynthesis of Sordarin}

To date, the biosynthesis of Sordarin has not been fully characterised and there are many steps which are not fully understood. However, one aspect that is known is that the metabolite cycloaraneosene is a key intermediate in this process and has been isolated from \textit{Sordaria araneosa}.\textsuperscript{135} Cycloaraneosene is believed to belong to a family of metabolites known as ophiobolins which share its 5-8-5 tri-cyclic core structure although there are some noticeable differences between them. Ophiobolins are notable in their own right due to their potent nematocidal activity and possible future use as anthelmintic drugs.\textsuperscript{136,137}

While there is a published biosynthetic route for the ophiobolins,\textsuperscript{138} it is not yet known if cycloaraneosene follows an equivalent or alternate pathway. However, there are several published total syntheses of cycloaraneosene.\textsuperscript{139-141}
biosynthesis is still largely unexplored, the cycloaraneosene intermediate and the likely benefit of the analysis of the biosynthetic pathway is that it provided a starting point for the biosynthetic route to sordaricin and eventually sordarin from cycloaraneosene is unknown. It has, however, has been proposed that the unusual tetracyclic core is formed via an intramolecular [4π+2π] cycloaddition reaction. While the exact route of sordarin biosynthesis is still largely unexplored, the cycloaraneosene intermediate and the likely [4π+2π] cycloaddition reaction have provided clues to its nature (Scheme 1). Another benefit of the analysis of the biosynthetic pathway is that it provided a starting point for attempts at total synthesis. These attempts may actually prove to be surprisingly analogous with the biosynthetic routes.

4.3 The Total Synthesis of Sordarin and Sordaricin
Along with the research into the sordarin as an antifungal agent there has been interest in using sordarin and sordaricin, as targets for total synthesis. If this could be accomplished in a low number of high yielding steps, it could be preferable to the biosynthesis. Also, the reproduction of the highly unusual [4π+2π] cycloaddition reaction believed to occur in the biosynthetic route is of great interest chemically.

4.3.1 Synthesis of Sordarins Tetracyclic Core via Unique [4π+2π] Cycloaddition Reaction
The very first reported attempt at a total synthesis was published in 1991 by Mander et al. The primary goal was to investigate the possibility of the [4π+2π] cycloaddition reaction presumed to occur during the biosynthesis of sordarin. It was initially thought that the yield could suffer from a [1,5]-sigmatropic shift followed by a [4π+2π] cycloaddition (Scheme 2).
**Scheme 2:** $[4\pi+2\pi]$ cycloaddition and competing rearrangement/cycloaddition reaction.

When a Diels-Alder reaction was tried on ketotriene 40, it produced only rearrangement/cycloaddition product 44. This result implies that the activation energy of the [1,5]-sigmatropic rearrangement is more favourable than the Diels-Alder reaction. To try and combat this effect, and reduce the activation energy of the Diels-Alder reaction, triene ester 41 was subjected to similar conditions. This proved partially successful, resulting in a ratio 43:45 3:2. However, this experiment resulted in 40% of unwanted by-product.

These results prompted the Mander group to design a synthesis of tetracyclic ester 53, which shares many core structural similarities to sordarin. In this synthesis, the $[4\pi+2\pi]$ cycloaddition is used as the key tricyclic ring forming reaction, but it is designed so interference via a [1,5]-sigmatropic shift is impossible.

This synthesis used compounds 46 and 47 as starting materials,\(^{144-145}\) which were coupled to produce methyl ester diene 48 (Scheme 3). The ester 48 was then reduced with Red-Al, methylated with methyl iodide and the acetal hydrolysed to produce 49. The keto group of 49 was then oxidatively cleaved via a Baeyer-Villiger reaction. The resultant carboxylic acid was lactonized to 50, and then subjected to a Vedejs hydroxylation to form 51.
Scheme 3: Synthesis of teracyclic ester 53; (i) LDA, HMPT; (ii) Red-Al; (iii) NaH, MeI; (iv) AcOH.H₂O; (v) H₂O₂, NaOH; (vi) BF₃·Et₂O; (vii) MoO₅·Py·HMPA; (viii) LiAlH₄; (ix) NaO₄; (x) DBU; (xi) toluene, reflux.

51 was then reduced to a lactol, cleaved with sodium periodate to form a dialdehyde, before the β-formyloxy aldehyde was cleaved to form 52 via heating with DBU. The [4π+2π] cycloaddition reaction was then performed successfully by refluxing 52 in toluene to produce the tetracycle 53. This reaction avoided the competing [1,5]-sigmatropic rearrangement due to the presence of a tertiary substituted carbon atom in the cyclopentadiene ring of 52.

This work was the first time the unusual tetracyclic core of sordarin was reproduced synthetically. Also, the [4π+2π] cycloaddition reaction pioneered in this synthesis provided the key step in many of the subsequent attempts at total synthesis.

4.3.2 Total Synthesis of Sordaricin Methyl Ester
The sordarin methyl ester 65 was successfully synthesised and published in 1993 by Kato et al. The key part of the retro synthetic plan devised by the Kato group was the formation of intermediate 54 (Scheme 4). This intermediate would allow the [4π+2π] cycloaddition reaction, detailed previously to be used to afford the tetracyclic core. Previously reported compounds 55 and enantiomerically pure 56 were used as starting materials for this synthesis.
Scheme 4: Retrosynthetic plan for the synthesis of sordaric.\textsuperscript{146}

The first step of the synthesis involved coupling 55 to 56 via the application CrCl\textsubscript{3} and LiAlH\textsubscript{4}, which was followed by the methylation of the newly formed hydroxyl group, forming bicyclic diene 57 (Scheme 5). After these initial steps, a Cope rearrangement was performed via heating to 200 °C in xylene producing compound 58. Compound 58 was then subjected to singlet oxygen promoted oxidation which converts the vinyl ether via an hydroperoxide intermediate and its subsequent elimination via acetyl chloride to afford an α,β-unsaturated methyl ester 59. The benzyl group is then cleaved with Pd(OH)\textsubscript{2} to form an alcohol 60, before it was region- and stereo-selectively hydrogenated using iridium black as a catalyst\textsuperscript{148} to form 61.

Scheme 5: Partial sordaric synthesis; (i) CrCl\textsubscript{3}-LiAlH\textsubscript{4}, DMF; (ii) NaH, Mel; (iii) Xylene, 200 °C; (iv) O\textsubscript{2}; (v) AcCl, Pyr; (vi) H\textsubscript{2}, Pd(OH)\textsubscript{2}; (vii) H\textsubscript{2}, Ir, tBuOH, 4 bar; (viii) Im, TBDMSI; (ix) (TMS)\textsubscript{2}NLi, MoO\textsubscript{3},Pyr complex, HMPA; (x) SOCl\textsubscript{2}, py-Ac\textsubscript{2}O; (xi) Bu\textsubscript{4}NF; (xii) (COCl\textsubscript{2})\textsubscript{2}, DMSO, Et\textsubscript{3}N; (xiii) (TMS)\textsubscript{2}NLi, TMSCl.
The hydroxyl group is then reprotected with TBDMSCl to give 62. Selective oxidation via MoO₃ catalyst and subsequent dehydration gave cyclopentane 63. Deprotection with tetra-n-butylammonium fluoride and oxidisation using Swern oxidation conditions and was finally trapped as trimethylsilylenolether 64, by Li(TMS)₂N and then TMSCl. Once 64 is formed, it is oxidised using Pd(OAc)₂ to form the α,β-unsaturated aldehyde 54 (Scheme 6). The [4π+2π] cycloaddition reaction was then performed by heating 54 to 40 °C in benzene, which produced the tetracycle 65. The methoxymethyl ether protecting group was then cleaved with acid, which formed the sordaricin methyl ester 66.

Scheme 6: Synthesis of sordaricin methyl ester 66; (i) Pd(OAc)₂; (ii) Benzene, 40 °C; (iii) AcOH-H₂O.

The total synthesis of the optically active methyl ester derivative of sordaricin 66 was achieved in 14 linear steps, from starting materials 55 and 56 in a 1.8% total yield. However, no attempt was reported to produce synthetic sordaricin 32 from methyl ester 66.

4.3.3 Synthesis of Sordaricin via a Novel Tandem Cycloreversion/Cycloaddition Reaction

In 2003 the first complete total synthesis of sordaricin was published by Mander et al.³ and was then revised and slightly improved in 2005, also by the same group. These syntheses were attempted with two goals in mind, the first of which was to find an efficient means esis for producing sordaricin. The second objective was to further analyse the chemically interesting tricyclic ring forming reaction.
Scheme 7: Strategy for total synthesis of sordaricin.

The strategy was slightly different to that previously published by the Mander group. The key intermediate in this synthesis is compound 68, which allows for investigation into a tandem cycloreversion/cycloaddition reaction, in addition to the \([4\pi+2\pi]\) cycloaddition that had been used previously (Scheme 7). It was proposed that, although the \([4\pi+2\pi]\) cycloaddition was proven to work effectively, the actual mechanism for the biosynthesis of the sordaricin core may be a tandem reaction rather than stepwise process.

Scheme 8: Partial sordaricin synthesis performed by Mander et al.,\(^{142}\) (i) LDA, HMPA; (ii) DIBAL-H, NaBH\(_4\); (iii) MOMCl, TsOH.

This synthesis started with a racemic mixture of 71, which was used to produce compounds 72 and 73 (Scheme 8). Compounds 72 and 73 were then coupled using an lithium di-isopropylamide in hexamethylphosphoramide (HMPA) to form 74. The nitrile 74 was then reduced to an aldehyde using DIBAL-H, and then further reduced to an
alcohol using NaBH₄. The newly produced hydroxyl was protected using MOMCl to produce ether 68. Ether 68 is the point where the synthesis of sordaricin via the cycloreversion/cycloaddition reaction deviates from the [4π+2π] cycloaddition method.

**Scheme 9**: Synthesis of sordaricin core 76; (i) LiHMDS, MeOCOCN; (ii) NaH, PhNTf₂; (iii) 2-Th(CN)CuLi, i-PrMgCl; (iv) MgBr₂.ET₂O, butanethiol; (v) MnO₂; (vi) 180 °C.

In the cycloreversion/cycloaddition synthesis (Scheme 9), the first step is the conversion of 68 to the enoate 75 via enolisation, C-acylation and coupling using a higher order cyano cuprate. The MOM protecting groups were then cleaved using MgBr₂.ET₂O and n-butanethiol, before the allylic alcohol was selectively oxidised to aldehyde 69. Aldehyde 69 then undergoes the cycloreversion/cycloaddition reaction. The mechanism for this reaction involves the initial loss of cyclopentadiene and then a [4π+2π] cycloaddition reaction. To force the cycloreversion, the reaction needed to be heated to 180 °C for 18 hours. However, these high temperature conditions resulted in a 4:1 ratio of sordaricin methyl ether 76 and an unwanted isomer 77. These isomers proved impossible to separate and resulted in the tandem cycloreversion/cycloaddition reaction being dismissed as a possible method for tetracycle formation in the biosynthetic pathway.
In the alternative synthesis of sordaricin (Scheme 10), the cycloreversion is performed on intermediate 68 using the same reaction conditions as the tandem cycloreversion/cycloaddition (180 °C, etc), which resulted in α,β-unsaturated pentanone 78.

![Chemical structures](image)

**Scheme 10**: Final synthesis of sordaricin; (i) 180 °C; (ii) LDA, MeO₂CCN, Et₂O; (iii) NaH, PhNTf₂; (iv) 2-Th(CN)CuLi, i-PrMgCl; (v) MgBr₂,Et₂O, butanethiol; (vi) MnO₂; (vii) 40°C, 3 days; (viii) NaSPr.

The synthesis then follows the same procedures as previously mention with the formation of isopropyl cyclopentadiene methoxycarboxylate 79, and alcohol deprotection, followed by selective oxidation to form 70. The [4π+2π] cycloaddition was then performed by heating 70 to 40 °C in benzene over a period of 3 days. This resulted in a single isomer of the sordaricin methyl ester, which was then demethylated using sodium 1-propanethiolate. This resulted in the first reported case of synthetic sordaricin being produced in 26 linear steps with an overall yield of 3%.
4.3.4 Synthesis of Sordaricin via a Tsuji-Trost Type Reaction

Scheme 11: Retrosynthetic analysis of sordaricin performed by Narasaka et al.\textsuperscript{149}

In May 2004 an alternative synthesis of sordaricin was published by Narasaka et al.\textsuperscript{149} which differed from the previous attempts by using Tsuji-Trost reaction as the key tetracyclic core forming step. The tricycle 81 was believed to be an intermediate which would enable a Tsuji-Trost reaction to form the tetracyclic core 80, which could then be converted into sordaricin (Scheme 11).

Scheme 12: Synthesis of key tricyclic intermediate 81; (i) [Mn(pic)\textsubscript{3}], n-Bu\textsubscript{3}SnH, 0 °C; (ii) AcOH, 50 °C; (iii) TBDMSI, Im; (iv) Me\textsubscript{2}NNH\textsubscript{2}, AcOH; (v) LDA, CH\textsubscript{2}=CHCH\textsubscript{2}Br, -78 °C; (vi) NaOAc, AcOH; (vii) OsO\textsubscript{4}, NMO, NaIO\textsubscript{4}; (viii) N\textsubscript{2}CHCO\textsubscript{2}Et, SnCl\textsubscript{2}, 35 °C; (ix) NaOEt, EtOH; (x) CH\textsubscript{2}=CHMgCl, CuBr.SMe\textsubscript{2}, TMSCl, -78 °C; (xi), Ac\textsubscript{2}O, DMAP; (xii) TsOH, 70 °C; (xiii) PCC; (xiv) CH\textsubscript{2}=CHMgCl, -78 °C; (xv) NaOEt; (xvi) TBSCI, Et\textsubscript{3}N, DMAP; (xvii) LDA, ClCO\textsubscript{2}Et, -78 °C; (xviii) TBAF.
Cyclopropanol 83 was used as a mix of diastereoisomers, in a 5-exo-trig ring closing reaction, affording bicyclic ketone 82 (Scheme 12). \(^{150,151}\) This reaction is an effective way of reproducing the three stereogenic centres associated with the methyl cyclopentane part of the sordaricin core. However, since cyclopropanol 83 was racemic, bicyclic ketone 81 exists as a mix of stereoisomers. The tetrahydropyran group was cleaved with acid and replaced using TBSCl to form ether 84. The regio and stereoselective allylation of 84 was achieved with three reactions, the first of which was the conversion of the ketone to a N,N-dimethyl hydrazone. The hydrazone was then allylated using lithium disopropylamide and allyl bromide, then a hydrolysis reaction afforded cycloheptanone 85. The vinyl group underwent a dihydroxylation using osmium tetroxide before being oxidatively cleaved to an aldehyde using sodium periodate. The aldehyde is then reacted with ethyl diazoacetate, using tin(II) chloride as a catalyst, to form \(\beta\)-keto ester 86.

\(\beta\)-Keto ester 86 was then cyclised via a Knoevenagel type cyclisation \(^{152}\) using sodium ethoxide, to form tricyclic compound 87. Tricycle 87 then undergoes a stereoselective 1,4-addition of vinylmagnesium chloride in the presence of CuBr.SMe\(_2\) and TMSCl. The resultant enol is then acetylated using acetic anhydride to produce tricyclic enol acetate 88. The TBS group was then removed using \(p\)-toluenesulfonic acid, to allow the hydroxyl to be oxidised to ketone 89 using pyridinium chlorochromate. Vinyl group addition was achieved using allyl magnesium chloride, before the acetate group was cleaved with sodium ethoxide. The hydroxyl group was then selectively reprotected using TBDMSCl to produce allylic alcohol 90. The hydroxyl is then converted to the ethyl carbonate via application of ethyl chloroformate, before the TBDMS group was cleaved using TBAF to form the tricyclic enol 81.
Scheme 13: Mechanism of tetracyclic core formation via Tsuji-Trost type reaction, performed by Narasaka et al.\textsuperscript{149}

The tetracyclic core 80 is then formed via a Tsuji-Trost reaction using catalytic amounts of [Pd(0)(PPh_3)_4] and sodium hydride (Scheme 13). The ketone is converted to an enol triflate (Scheme 14) by the application of LDA and N-(5-Cl-2-pyridyl)triflimide. This is followed by the addition of a higher order cuprate to produce compound 91. The two vinyl groups of compound 91 were first oxidised to 1,2-diols with osmium tetroxide and then esterified with phenylboronic acid, to form bis-phenylboronic ester 92. The two phenylboronic esters were then oxidatively cleaved with sodium periodate to form the dialdehyde 93.
The dialdehyde 93 was then reduced to a diol using sodium borohydride before the selective protection using TBDMSCl afforded compound 94. The free hydroxyl was then oxidised to an aldehyde using sulphur trioxide-pyridine complex. The TBS and ethyl ester groups were then cleaved using TsOH and propanethiolate respectively, which resulted in synthetic (+/-) sordaricin 32. Overall, the synthesis was performed in 27 linear steps in a yield of 5%.

4.3.5 Total Synthesis of (-)-Sordarin

The first and, to date, only synthesis of (-)-sordarin was published in 2006, by Narasaka and Chiba et al.\textsuperscript{153} This paper is a continuation of the work detailed in Section 4.3.4, also performed by Narasaka and Chiba et al. The plan was to alter the original method slightly to stereoselectively form (-)-sordaricin 32 and then with the addition of a synthetic D-sordarose derivative, form (-)-sordarin 31.
Scheme 15: The stereoselective synthesis of bicyclic ketone 84; (i) CuBr.SMMe2, TMSCl, HMPA, -78 °C; (ii) Et3Zn, CH2I2, Et2O, reflux; (iii) K2CO3, MeOH; (iv), AgNO3, (NH4)2S2O8, Py, 1,4-cyclohexadiene, DMF.

In the 2004 synthesis, the Narasaka group used cyclopropanol 82 with a mix of diastereomers present (Scheme 12) which eventually led to the formation of (+/-)-sordaricin. In the 2006 synthesis, the Narasaka group used optically pure 95154 as a starting material (Scheme 15). This starting material was then treated with 3-butenylmagnesium bromide 96 and trimethylsilyl chloride (TMSCl) in the presence of CuBr.SMMe2 to form the silylenol ether 97. Silylenol ether 97 then underwent cyclopropanation using diethylzinc and diiodomethane, before the TMS group was cleaved using potassium carbonate. The resultant cyclopropanol 98 was then subjected to a modified version of the oxidatitive 5-exo-trig ring closing reaction detailed in Section 4.3.4. In this reaction silver nitrate, (NH4)2S2O8, pyridine and 1,4-cyclohexadiene was used to form bicyclic ketone 84 stereoselectively. Due to optically pure 95 being used as a starting material, the synthesis of bicyclic ketone 84 produced a single enantiomer.

Scheme 16: Alternative synthesis of tricyclic 87; (i) Me2NNH2, AcOH; (ii) LDA, 100, -78 °C; (iii) AcOH, NaOAc; (iv) EtONa, 60 °C.
Although the 2006 synthesis followed the 2004 synthesis almost exactly from bicyclic ketone 84, there was a simplification of the formation of tricyclic compound 87 (Scheme 16). The ketone 84 was still converted to a N,N-dimethylhydrazone 99 and then allylated, but this time with using lithium diisopropylamide and 6-bromo-2,2-dimethyl-1,3-dioxin-4-one 100. The hydrazone was then converted back to a ketone using acetic acid and sodium acetate to form tricyclic ketone 101. The tricyclic ketone 101 was then treated with sodium ethoxide, cleaving the 1,3-acetonide group and allowing a condensation reaction to occur. These successive reactions form tricyclic ethylester 87 in four reactions, as opposed to the six in the original 2004 synthesis. Sordarin 32 was then synthesised 87 as detailed in Section 4.3.4, with very minor modifications to catalysts, solvents etc.

The second part of the synthesis was the construction of a D-sordarose derivative. This synthesis was complicated by the need to have an attachment strategy which would control the C-1 stereogenic centre of the glycoside. The abundant carbohydrate D-mannose was chosen as the starting material, as the stereogenic centres at C-2, C-4 and C-5 are identical to that of the D-sordarose.
Scheme 17: Synthesis of a synthetic glycoside 114, (i) LiAlH₄, THF, reflux; (ii) TBAF, THF, (iii) NaH, MeI, DMF; (iv) TsOH·H₂O, MeOH, 50 °C; (v) Bu₂SnO, Toluene, reflux; allylBr, Bu₄NI, DMF, reflux; (vi) NaH, 4-MeOBrCl, Bu₄NI, DMF, 50 °C; (vii) RhCl(PPh₃)₃, DABCO, EtOH·H₂O, reflux; OsO₄, NMO, acetone; (viii) DMP, NaHCO₃, DCM; (ix) NaBH₄, MeOH, 0 °C; (x) PMBzCl, DMAP, pyr; (xi) NBS, acetone·H₂O; (xii) DAST, DCM.

D-Mannose was first protected via previously developed methodology to 104.¹⁵⁵ The C-6 tosylate group was reductively eliminated using lithium aluminium hydride to form the 6-deoxy sugar. The triisopropylsilyl (TIPS) group was cleaved using TBAF before the hydroxyl was methylated using methyl iodide to form 105. The acetonide group was then removed using TsOH before the C-3 hydroxyl was selectively allylated using allyl bromide and a dibutyltin oxide catalyst. The C-2 hydroxyl of glycoside 108 then protected using a PMB ether, before the allyl group was cleaved using tris(triphenylphosphine)rhodium(I) chloride and oxidatitive cleavage using osmium tetraoxide. The C-3 hydroxyl group of 109 was then successfully inverted via oxidation-reduction sequence to 111. Several different bulky groups were attached to the C-3 hydroxyl group at this point in the synthesis, in an attempt to control the stereochemistry of the final glycosylation. Of the groups tested, the PMB ester 112 appeared to offer the
best stereocontrol. The phenylthio group was converted to the more reactive glycosyl fluoride 114 using a sequential treatment of N-bromosuccinimide followed by DAST.

Scheme 18: Synthesis of sordarin 31; (i) AgClO₄, SnCl₂, Et₂O; (ii) DDQ, DCM; (iii) EtONa, EtOH; (iv) n-PrSNa, HMPA.

The glycosylation was then performed using Mukaiyama conditions, which afforded a 6.5:1 ratio of the anomeric products in favour of the β-anomer 117 (Scheme 18). It is proposed that this selectively is due to 1,3-anhichiamer assistance from the PMBz group (over riding the potential for assistance 1,2-anhichiamer from the PMB group) and the steric effects of using sordarin ethyl ester 115 as the nucleophile. The PMB and PMBz groups are then cleaved using DDQ and sodium ethoxide respectively. In the final step the ethyl ester is cleaved using propanethiolate to produce the carboxylic acid and afford (-)-sordarin 31.

4.3.6 Summary of the Total Syntheses of Sordarin and Sordaricin

The defining features of these three routes to synthesise sordaricin are their unique approaches to the formation of the tetracyclic core. The unique features of sordarin represent a challenge to its total synthesis and to meet this challenge, three unique
methods were developed. The first method was the $[4\pi+2\pi]$ cycloaddition reaction which, as mentioned in Section 4.2, is thought to be the most likely biosynthetic route for the formation of sordarin. This reaction was performed in both the 1991 and 1993 syntheses by the Mander and Kato groups respectively. The Kato group managed a yield of 58% from this reaction while the Mander group achieved a successful reaction in an 80% yield. In the 2003 synthesis this was further improved with the $[4\pi+2\pi]$ cycloaddition reaction quoted as having a 100% yield. This reaction appears to have been a good choice for the formation of the tetracyclic core, as the yield was not only very high but there were no competing isomers observed from these reactions.

Also in the 2003 synthesis, the Mander group attempted to shorten the overall route by attempting a tandem cycloreversion/cycloaddition reaction which had a respectable yield of 76%. However, it resulted in isomers being formed in a 4:1 ratio in favour of the sordaricin methyl ester 76. The isomers 76 and 77 proved impossible to separate, which led the Mander group to disregard this strategy as an effective way of forming the tetracyclic core.

The third method for tetracyclic core formation used is the Tsuji-Trost reaction, which was performed by Narasaka et al. This reaction ultimately proved highly successful with a yield of 92% and produced no observable isomers.

In summary, sordaricin has been successfully obtained via three routes with overall yields of 2% for the Kato group, 3% for the Mander group and 7% for the Narasaka group. Later the Narasaka group reported sordarin formation in a 5% yield. However, perhaps unsurprisingly, all these synthetic routes have far too many reaction steps to be considered as a viable alternative to biosynthesis.

4.4 Antifungal Derivatives of Sordarin

As previously mentioned sordarin is a known to be an antifungal agent, this has led to an interest in improving on this activity. The majority of this research revolves around
cleaving the D-sordarose and attaching a variety of different compounds to the hydroxyl. In almost all cases, the aglycone, sordaricin, is more or less untouched. We are more interested in methodologies which have been developed which can be used to produce a directed library of sordarin derivatives using commercially available material.

### 4.4.1 Synthesis of a Library of Sordarin Derivatives via the use of Lithiated Reagents

A library of keto and keto-alkyne sordarin derivatives was created via methodology developed by Quesnelle et al.\(^{156}\) The basis of this method was to lithiate simple iodinated alkyl and alkyne compounds, then use these lithiated regents as competent nucleophiles. The carboxylic acid and aldehyde groups would obviously be ideal for this kind of nucleophillic addition. However, previous investigations had indicated that any interference with the carboxylic acid and aldehyde groups of the aglycone led to a decrease in antifungal activity. With this in mind, a synthetic plan was developed which would first cleave the D-sordadrose and then protect the carboxylic acid and aldehyde groups (Scheme 19). The hydroxyl group could then be oxidised to an aldehyde, which would be an ideal target for nucleophillic attack using the lithiated reagents.

![Scheme 19: Formation of tetracyclic aldehyde](image)

The glycoside was successfully cleaved via acid hydrolysis with hydrochloric acid (Scheme 19). The carboxylic acid was then protected as a p-methoxybenzyl ester and the aldehyde as an acetonide. The C-8 hydroxyl group was then oxidised to an aldehyde under Swern conditions, affording tetracyclic aldehyde 119.
A range of iodinated alkyl and alkyne compounds were lithiated via application of s-BuLi to form a library of nucleophiles (Scheme 20). These nucleophiles were then applied to the tetracyclic aldehyde 119, at -78 °C, in tetrahydrofuran, to produce a library of sordarin derivative secondary alcohol. These compounds were then oxidised back to restore the ketone functionality, also under Swern conditions, before the C-4 aldehyde was unveiled. In the final step the PMB ester was cleaved via hydrogenolysis.

**Scheme 20**: Sordarin derivative library synthesis via nucleophilic addition; (v) s-BuLi, THF, -78 °C; (vi) THF, -78 °C; (vii) DMSO, (COCl)₂, Et₃N, DCM, -78 °C; (viii) HCl, MeOH; (ix) Pd(OH)₂, H₂, EtOH.

A large library of sordarin derivatives were produced using this methodology and were tested for antifungal activity against *Canadia glabrata* and *Canadia albicans*. These assays found cyclopentane 120 and isopropyl 121 were highly effective against both strains. It was noted that in general the alkyne derivatives tended to have better activity than the simple alkyl groups.

**4.4.2 Synthesis of Sordarin Derivatives**

The alkyl ether derivatives of sordarin were synthesised by Tse and Kaneko *et al.*,¹⁵⁷,¹³³ and found to have a superior pathogenic fungi activity than either sordarin or sordarin.
Scheme 21: Production of cyclohexane sordarin derivatives; (i) BnBr, NaHCO₃, DMF; (ii) Tf₂O, Py/DCM; (iii) mercaptocyclohexane, NaH, DMF, (iv) H₂, Pd(OH)₂-C, MeOH; (v) N-methyl cyclohexylamine, BuLi, THF; (v) cyclohexanone oxime, KH, DMF (vi) CH(OCH₃)₃, (CH₂OH)₂, TsOH, MeOH; (vii) (COCl)₂, Me₂SO, DCM, Et₃N; (viii) cyclohexanol, Ph₃P, DEAD, DMF; (ix) 2,4,6-triisopropylbenzenesulfonyl chloride, Et₃N, DMAP, DCM, cyclohexylamine; (x) HCl, MeOH, H₂, (xi) C-Pd(OH)₂, MeOH; (xii) 2,4,6-triisopropylbenzenesulfonyl chloride, Et₃N, DMAP/DCM, cyclohexylamine.

During the initial investigation a cyclohexyl ether derivative 127 was found to have strong activity. A directed library of cyclohexyl ring containing sordarinic derivatives were synthesised, including examples of ethers, esters and amines (Scheme 21). All of the derivatives together with sordarin and sordarinic as controls, were tested for activity against Canadia glabrata ATCC90030, Canadia tropicalis ATCC750 and multiple strains of Canadia albicans. Interestingly, of all the compounds tested the cyclohexylthio derivative 123 was found to have superior antifungal activity.
5.0 Sordarin as a Splicing Inhibitor

When this project was first conceived, it was in collaboration with Diversa, a US-based pharma company. Diversa had agreed to provide the sordarin needed for this project. However, the company has since been taken over and the research they were performing on sordarin seems to have been discontinued. Hence, the biosynthesis and isolation of sordarin directly from *Sordaria araneosa* was attempted. These cultivations were performed by Dr Lilly Novak-Frazer from the Faculty of Life Sciences, University of Manchester, a member of the O’Keefe research group. The O’Keefe group are largely responsible for our current knowledge of Snu114 and a co-collaborator on this project.

5.1 Cultivation and Isolation of Sordarin

Previously, sordarin has been of interest due to its antifungal activity and a number of research groups have attempted to optimise the biosynthesis. The interest in optimisation of the biosynthesis appears to be due to the relatively small quantities of sordarin produced from large fermentations. This low yield represents a challenge when producing the larger quantities of sordarin needed if a prototypical drug of any kind was to be developed.

The method used here was a slight modification of that method developed by Tully *et al.* Their attempted optimisation of sordarin production was part of a greater attempt by Bristol-Myers Squibb to identify novel structures exhibiting antifungal activity, particularly against the genera *Cryptococcus* and *Aspergilla*. These experiment were performed on *Sordaria araneosa* (ATCC 36386) and were cultivated on Emerson YSS agar (15.0 g soluble starch, 4.0 g yeast extract, 1.0 g K₂HPO₄, 0.5 g MgSO₄·7H₂O, 20.0 g agar per litre distilled water) at 25 °C for 15 days.

During these fermentations of *Sordaria araneosa*, two variables were found to be vitally important in the production of sordarin. The first was the high temperature sensitivity of sordarin growth. To date, the only growth observed for sordarin is when *Sordaria*
araneosa was fermented at 25 °C for the duration the growth. Other experiments conducted at other temperatures, e.g. 23 °C and 27 °C, failed.

The second variable was the incubation period. An assessment was made of the growth pattern of sordarin, using HPLC and LC-MS techniques. This appeared to show that 15 days was the optimal incubation period, although the stability of sordarin in media for longer periods is unclear. Despite all attempts to optimise sordarin growth, to date, the cultivation of sordarin is still temperamental.

5.2 Sordarin Extraction

Once the cultivation stage has been completed, sordarin is extracted from the media. This can be achieved via two different methods, the first of which was developed by Yau et al.161 In the first method a polyaromatic adsorbent resin known as Diaion HP-20 was added to the sordarin cultivation and stirred overnight. The resin is then washed with a mix of acetone and water before being evaporated in vacuo, producing a crude residue containing multiple biomolecules. An alternative method was tried in which the mycella is filtered off before the aqueous solution is extracted three times with an equivalent volume of ethyl acetate. This is then evaporated to dryness in vacuo, also producing a crude residue. However, analysis via LC-MS indicated fewer impurities present when the Diaion resin extraction method was used. Once extracted the crude material is first purified using a traditional silica column and then purified again using HPLC.
The literature states that it is possible for sordarin ‘like’ compounds to be produced from the cultivation such as hypoxy-sordarin 129 and neo-sordarin 130. However, these compounds have rarely been found in cultivations using *Sordaria araneosa* ATCC 36386 strain, which were used here. As expected, HPLC analysis indicated that these compounds have not been produced in any of the experiment conducted here in Manchester so far.

Overall, the success of these cultivations was highly variable with most producing no usable sordarin material. To date, the most successful cultivation produced 24 mg of pure sordarin, which had $^1$H and $^{13}$C NMR values equivalent to literature values.

### 5.3 Sordarin Derivatives

Due to the shortage of sordarin produced from the biosynthesis, the number of derivatives that could be formed where unfortunately limited. The first reaction involved the methylation of sordarin via the application of diazomethane in a 2:1 toluene: methanol mixture as solvent, which afforded methyl ester 131 in excellent yield.
Scheme 22: Formation of sordarin 32 and derivative 131; (i) TMSCH$_2$N$_2$, MeOH/Toluene; (ii) HCl, EtOH.

The glycoside was cleaved via an acid catalysed hydrolysis reaction, in which sordarin was stirred with hydrochloric acid in acetone over a period of 48 hours. Although slow, this reaction afforded sordarin 32 in a high yield.

5.4 Splicing Assay Results for Sordarin Derivatives

Sordarin and its derivatives were tested for splicing inhibition activity in both human and yeast cells (Figure 21). These assays used either; a) 4 mM of inhibitor, in a 4% DMSO solution, in yeast or b) 5 mM of inhibitor, in a 1% DMSO solution, in human cells. These assays were performed by Ray O’Keefe from the Faculty of Life Sciences, University of Manchester.

The results obtained from these experiments show that sordarin inhibits pre-mRNA splicing in both yeast and human cells, with a significant decrease in the formation of both mRNA and the intermediate products formed in splicing (lariat intron, etc). The methyl ester 131, showed higher concentrations of mature mRNA formed in both yeast and human cells, when compared to sordarin. The activity shown for methyl ester 131 is actually comparable to that seen in the dimethyl sulfoxide control sample, indicating that methyl ester 131 has almost no activity. Sordaricin 32 actually had a lower concentration of mature mRNA being formed, than sordarin 31.
Figure 21: (A) Splicing of ACT1 pre-mRNA for 20 min in yeast whole cell extract with 4 mM sordarin or its derivatives in 4% DMSO, (B) Splicing of β-globin pre-mRNA for 2 hrs in HeLa nuclear extract with 5 mM sordarin or its derivatives in 1% DMSO.

These results prove that both sordarin 31 and sordaricin 32 are splicing inhibitors in both human and yeast cells. These results are significant because sordarin selectively inhibits EF-2 in fungi and not in human cells,\textsuperscript{126} therefore the inhibition of human splicing by sordarin was far from assured. The decrease in activity shown by the methyl ester 131 indicates the carboxylic acid functionality is vitally important to the activity of sordarin 31. These results are in accord to the results found when the equivalent derivatives were tested for antifungal activity.\textsuperscript{10,83,148,162} In the [EF-2:sordarin] X-ray structure,\textsuperscript{127} the carboxylic acid is shown to bind to the backbone amide of glutamic acid residue 524 (Figure 20). This glutamic acid residue is conserved in Snu114 and hSnu114 (Figure 19), implying that sordarin could bind to the equivalent Snu114 residue. The increase in activity shown when the aglycone, sordaricin 32, was used indicates that the glycoside actually had a negative effect on the activity of sordarin. As previously mentioned in Section 3.5, the two binding sites known to associate with the glycoside in the [EF-2:sordarin] complex are not conserved in either yeast Snu114 or hSnu114. Therefore, this result appears to back up the initial hypothesis that growth factor EF-2 is a close homologue to Snu114, or at least shares a similar binding domain.
Although few sordarin derivatives could be screened, the results obtained are valuable. They appear to corroborate the initial hypothesis of Snu114 and EF-2 homology. What is quite surprising is how closely the predictions made from the simple homology model appear to match the results found. It was initially predicted that the binding to the aglycone of sordarin would be similar in both Snu114 and EF-2. The binding to D-soradrose to Snu114 was predicted to not be as effective, and both of these predictions proved to be the case. The binding site associated with the acid was also predicted to be conserved in Snu114. Therefore, the carboxylic acid was predicted to be an important splicing factor and results appear to back this up also. It is also interesting to note that both these results appear to correlate with those obtained by other research groups investigating sordarins antifungal activity. As previously mentioned, the carboxylic acid functionality of sordarin is essential to the antifungal activity, with all modifications to the acid functionality to date, leading to a decrease in activity.\(^{10,83,148,162}\) In antifungal studies, sordaricin 32 has a slightly lower activity than sordarin 31, while in splicing, the activity is much increased.

This extra activity is explained by the lack of conserved binding sites from EF-2. However, it is interesting to note that, to date, the most successful sordarin derivatives produced have left the aglycone relatively intact and merely substituted at the C-8 hydroxyl group.\(^{133,156,163}\) These results imply that it might be possible to further improve the splicing activity of sordaricin by adding a functional side chain to the hydroxyl to better suit the different environment in yeast Snu114 and hSnu114, compared to EF-2.

### 5.5 Glycoside Replacement Strategy

As reported in Section 5.6, the cleavage of D-soradrose residue of sordarin to form sordaricin 32 led to an improvement in splicing inhibition. This observation implies that the glycoside is not well tolerated within the binding pocket. However, this does not rule out replacement glycosides being beneficial to the splicing inhibition of sordarin derivatives.
5.6 Production of Synthetic Glycals

5.6.1 Production of Synthetic Glycals via Commercially Available Glycosides

Initially, the synthesis of these glycals was achieved using commercially available glycosides, such as D-glucose 132 (Scheme 24). The first step in this process was the acetylation of the hydroxyl groups via acetic anhydride and sodium acetate. Pentaacetate 133 was then α-brominated with hydrogen bromide in acetic acid, before a zinc mediated 1,2-elimination reaction afforded 3,4,6-triacetate glucal 135.

Scheme 24: The production of 3,4,6-triacetate glucal 135; (i) Ac₂O, NaOAc; (ii) HBr, AcOH; (iii) Zn, Melm, EtOAc, reflux.

This process produces stereochemically pure material without the need for resolution, chiral catalysts or enzymes. This sequence of reactions was used for the following commercially available carbohydrates: D-galactose, D-arabinose, D-fucose, and D-xylose.
As the reaction sequence was straightforward a further discussion of the chemistry is not warranted, however, the full experimental methods are given in Chapter 9.

5.6.2 Synthesis of 4,5-Dihydro-pyran-3-ones via a Hetero Diels-Alder Reactions

Using naturally occurring carbohydrates to synthesise glycals ensures chiral purity, but is unfortunately limited in scope. Ideally, synthetic 4,5-dihydro-2H-pyran rings could be produced with points of diversity built into the methodology. The method chosen to achieve this objective was via a hetero Diels-Alder reaction involving a 1,3-diene and a variety of aldehydes, catalysed by Lewis acids. This methodology would allow for the production of synthetic 4,5-dihydro-pyran rings with a point of diversity at C-5.

Scheme 25: Synthesis of 3,3-dibromo-1,1-binapthol 140; (i) FeCl₃.6H₂O, toluene; (ii) (R,R)-DACH, toluene, crystallisation and isolation of (R) an (S)-137; (iii) MOMCl, THF, N₂, 0 °C; (iv) (CCl₃Br)₂, nBuLi, THF, 0 °C; (v) Amberlyst 15, THF, MeOH, N₂, reflux; (vi) Et₂Zn, N₂, DCM.
The problem with this methodology is that under normal Lewis acid catalysed conditions it would produce a racemic mixture. This can be solved via the use of a chiral catalyst. 3,3-Dibromo-1,1-binaphth-2-ol/zinc complex 141 was selected for this role, due to this catalyst having one of the highest published enantioselectivity values for a hetero Diels-Alder type reaction, whilst using a relatively simple ligand type. 164 3,3-Dibromo-1,1-binaphth-2-ol 140 was successfully synthesised in five steps (Scheme 25). Two naphthol 136 molecules were oxidatively coupled using an iron chloride catalyst, affording 1,1-binaphth-2-ol 137. Resolution of the two binaphthol enantiomers was achieved by complexation with (R,R)-1,2-diamino cyclohexane in an approximate overall yield 60% (30% each for the (R) and (S) enantiomers). 165 Methoxymethyl ether 138 was then produced via protection of the hydroxyl groups with chloromethylether. The next step involved the regioselective dibromination of the C-3 positions of bis-MOM ether 138 using a directed lithiation strategy. The dilithio anion was quenched with 1,2-dibromo tetrachloroethane to produce bis-MOM ether 139. The final step involved the cleavage of the bis-MOM ether group with Amberlyst-15 resin, affording 3,3-dibromo-1,1-binaphth-2-ol 140.

Once 140 was synthesised, it was used as a ligand in chiral catalyst 141. The complex 141 is formed by mixing equimolar amounts of diethyl zinc and 140 under inert atmosphere. The zinc acts as a Lewis acid catalyst for the hetero Diels-Alder reaction, reducing the energy of the aldehyde. This allows for more efficient overlap compared to the LUMO of the diene, which in turn reduces the activation energy enough to allow the reaction to take place. The mechanism for the enantioselectivity of the catalyst involves (R)-141 sterically hindering the approach to the si-face of the aldehyde. This effect then forces the diene to attack the re-face of the aldehyde forming the 5-(R)-enantiomer of 144.
Scheme 26: Hetero Diels-Alder reaction using Danishefsky's diene and benzaldehyde.

This reaction was tested using benzaldehyde under various conditions in an attempt to repeat the Wu groups results. The conditions found to produce the best results were using dry DCM at 0 °C and under an inert argon atmosphere. This resulted in a 75% enantiomeric excess (ee) of that (R)-144, as measured by chiral HPLC. This result demonstrates a useful level of selectivity but is still a significant reduction from the value quoted by Wu et al.\textsuperscript{166} Through a series of control reactions it was discovered that the loss of ee is probably due to unbound diethyl zinc forming racemic products, together with a loss of dryness within the reaction vessel over time. Although this enantiomeric excess was not as much as we would like, it was sufficient for our purposes, this is due to the fact when coupled to sordarin it would form distereomers and likely be isolatable.

5.7 Methods for Glycosidation
Two different methods were chosen for attachment of the sordaricin 32 to the glycols. The first method chosen was a triphenylphosphonium bromide mediated glycosylation while the second method was a phosphoric acid mediated Ferrier rearrangement. This methodology was tested using D-glucal triacetate 135 and 2,2-dimethylpropanol 145 as a sordaricin substitute.
The nucleophilic addition was tested first using triphenylphosphonium bromide crystals as a catalyst. This reaction afforded a 1:4 mixture of $\beta$:$\alpha$ anomers (146 and 147) in a total yield of 49% (Scheme 27). It has been shown previously that the ratio increases in favour of the $\alpha$-anomer when the size of the alcohol used is increased.167 This implies that sordarin would be more selective for the $\alpha$-anomer than 145, which is actually the complimentary stereochemistry to that found in sordarin 31.

The second method is a Ferrier rearrangement of 135 using phosphoric acid as a catalyst. This reaction was chosen as it provides an excellent yield, with the catalyst being extracted without the need for column chromatography.168 A model reaction was performed using the same 2,2-dimethylpropanol 145 and glucal triacetate 135 used for the nucleophilic addition. This reaction afforded 2,3-dihydro diacetate 149, in a 4:1 ratio of the $\alpha$:$\beta$ anomers (148 and 149), in an overall yield of 58%.

### 5.8 Glycoside Replacement

Unfortunately, due to the lack of sordarin produced from the biosynthesis of *Sordaria araneosa* these reactions could not be performed on sordarin itself. If a new source of sordarin can be found then it should be a simple task to react the already synthesised glycals with sordarin 32 to synthesise a library of sordarin derivatives. With five available glucals, two reactions and a possible two anomers for each reaction, a possible 20 sordarin derivatives could be synthesised from this small library alone.
Figure 22: Possible library of sordarin derivatives made by proposed nucleophilic addition or Ferrier rearrangement of glycals. (Figure shows only α-anomer but screening quantities of the β-anomer should be available from this methodology)

These products should provide valuable information on whether a 6-membered heterocycle can be accommodated within the binding pocket. Previous research analysing the antifungal activity via replacement of the glycoside has led to some of the more successful inhibitors.\textsuperscript{163,169} Continuation of this research would allow us to discover if this trend in antifungal activity correlates to splicing inhibition activity.

5.9 Proposed Disulfide Library Synthesis

One of the most commonly used methods for synthesising a diverse library of compounds is via thiol coupling reactions.\textsuperscript{170-174} These coupling reactions have been used to form libraries of disulfide compounds quickly and efficiently from commercially available thiols.\textsuperscript{175} For this methodology to work for sordarin, a thiol group would have to be introduced.
5.9.1 Proposed Thiol Addition

A good method for introducing a thiol group is via the displacement of a hydroxyl group. A two-step process using firstly diethyl azodicarboxylate and triphenylphosphine to afford a thio acetate derivative, and secondly hydrolysis to give the free thiol, has been established by Forsyth et al (Scheme 28).  

$$\text{R}^{\text{SH}} \text{OH} \xrightarrow{\text{Ph}_3\text{P}, \text{AcSH}} \text{R}_\text{SAc} \xrightarrow{\text{LiOH}, \text{MeOH}, \text{H}_2\text{O}} \text{R}^{\text{SH}}$$

Scheme 28: Proposed method for thiol addition.

This reaction would afford a thioacetate which could then be deacetylated to a thiol via application of lithium hydroxide. The hydroxyl to thiol group interconversion was proven to be highly efficient by the Forsyth group, making it ideal for the application of introducing a thiol group onto the sordaricin core.

Scheme 29: Proposed routes for the introduction of a thiol group to the tetracyclic core of the sordaricins. (i) TMSCH$_2$N$_2$, Tol-MeOH; (ii) NaBH$_4$, CeCl$_3$, MeOH; (iii) Ph$_3$P, AcSH, DIAD, THF; (iv) LiOH, MeOH-H$_2$O; (v) HCl, acetone.
Two methods are proposed for the introduction of a thiol group onto tetracyclic core of sordaricins (Scheme 29). The first method is a direct thiol replacement of the hydroxyl group exposed when sordaricin is formed, affording 163. This proposal continues the premise that replacement of D-sordarose is one of the more promising leads in the search for superior splicing inhibitors.

The second method, affording 161 and 162, would leave the glycoside intact, instead the aldehyde would be reduced to a hydroxyl and then converted to thiol. This could in theory work with both sordarin and sordaricin providing that the hydroxyls were protected. Since results show that the splicing inhibition for sordarin is not as high as sordaricin, it would seem that the formation of the sordaricin thiol derivative 162 would be preferable.

5.9.2 Proposed Oxidative Disulfide Formation

The sordarin thiol derivatives could then be coupled to commercially available thiols, thus producing a library of disulfide compounds. An environmentally benign and easily purified method for producing disulfides has been developed by Hirai et al. This method involves reacting the a thiol derivatives with commercially available thiols in the presence of a catalytic amount of iodine and hydrogen peroxide.

Scheme 30: Proposed method for disulfide coupling.
It should therefore be possible, using the methodology described, to introduce thiols to the sordaricin core at C-19 and C-20, thus forming two separate disulfide libraries (Scheme 30). If successful, this would be an efficient way of assessing the binding pocket environment around C-19 and C-20, and would provide insight for the on-going search for improved splicing inhibition.
6.0 Fusidic Acid

Due to the quantity of sordarin obtained the number sordarin derivatives produced was unfortunately limited. This caused us to go back to our initial hypothesis and search for other small molecule inhibitors. As described in Section 3.3, EF-G is also a close homologue of Snu114. Although, there is weaker homology between EF-G and Snu114 than EF-2 and Snu114, EF-G can be inhibited by fusidic acid. Accordingly, the next Section details fusidic acid and our screening experiments.

6.1 Fusidic Acid and EF-G inhibition

Fusidic acid belongs to a small family of compounds known as the fusidanes and was first reported in 1962 by Godfrensen et al.\textsuperscript{178} later in the same year the structure of fusidic acid \textsuperscript{166} was published.\textsuperscript{179} However, due to limitations in analytical techniques at the time, the C-11 hydroxyl group was mis-assigned as a C-12 hydroxyl group, the stereochemistry at C-16 incorrectly assigned and many of the stereocentres were left undefined. These problems were addressed later and the structure was assigned via rigorous chemical testing,\textsuperscript{180} but due to the complexity of the molecule full NMR assignment was not achieved until 2002.\textsuperscript{181} The conformation of fusidic acid is unusual compared to the conventional steroidal core. Fusidic acid has a \textit{trans-syn-trans} stereochemical arrangement and adopts a chair-boat-chair conformation as illustrated in Figure 23.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fusidic_acid.png}
\caption{The structure, conformation and first structural assignment of fusidic acid 166.}
\end{figure}
Fusidic acid is best known as an orally active antibiotic which was discovered when it was first reported by Godfrensen et al., the reason for this antibiotic activity was revealed to be its inhibition of elongation factor EF-G. The inhibition is as a result of formation of a stable ribosome:fusidic acid complex. This complex comprises fusidic acid, EF-G and GDP; and this formation causes an arrest of the ribosomal function. Fusidic acid has also been found to partially inhibit the ribosomal elongation factor EF-2. 182, 183

![Figure 24](image)

**Figure 24**: X-ray crystallography of [EF-G:GDP:166] complex with fusidic acid (purple), shown in relation to GTP (light green).

### 6.2 Conservation of Amino Acid Residues that bind Fusidic Acid, from EF-G to Snu114

Like EF-2, fusidic acid binds within the G-domain of EF-G. Sordarin binds to EF-2 within its own G-domain. However, although both molecules bind within the G-domains of their respective target proteins, the specific binding sites are very different and show no obvious homology. Four out of the eight amino acids known to contact fusidic acid are completely conserved between EF-G and hSnu114. Three of the eight amino acids are conservatively changed (Figure 25), Ile21 and Ile65 are both replaced by Leu and His458.
being replaced by Tyr. However, Thr437 is replaced by glycine, which is a significant change. In the binding site, yeast Snu114 shares many of the same features of hSnu114 with the exception of Thr84 and Thr437. Thr84 is conserved between EF-G and hSnu114, but is replaced by an alanine in Snu114, while Thr437 is changed to Gly in hSnu114 but is actually only moderately changed to Ser in Snu114.

![Diagram showing comparison of G domains of ribosomal growth factor EF-G with yeast and human Snu114 cells](image)

**Figure 25**: Comparison of G domains of ribosomal growth factor EF-G with yeast and human Snu114 cells (Dark blue = complete homology, light blue = similarity, white = no homology, arrows = known fusidic acid contacts).

Thr437 is especially important, as this amino acid interacts with the C-3 hydroxyl of the bound fusidic acid 166 (Figure 26). This lack of conservation implies that the hydroxyl at C-3 may not contribute significantly to the binding of fusidic acid to Snu114 or may even impact negatively on the binding. Modifications to the C-3 hydroxyl of fusidic acid may be a useful tool to differentiate between EF-G, Snu114 and hSnu114.
As previously mentioned, the only differences in the retention of binding sites between Snu114 and hSnu114 is Thr84 and Thr437. Thr84 appears to bind to the acetate group situated at C-16 in fusidic acid. This implies there may be a discrepancy between the splicing inhibition activity of fusidic acid and its derivatives in human and yeast cells. This difference could possibly be magnified if reactions are performed which modify the C-16 functionality. As mentioned previously the change of Thr437 to glycine in hSnu114 could negatively affect binding, however in Snu114 this change is conservative with Thr being replaced by Ser. This difference in homology could provide a significant difference in the ability of fusidic acid homologues to bind to Snu114 and hSnu114.

### 6.3 Fusidic Acid Biosynthesis

A known precursor of fusidic acid is squalene 173, which is itself synthesised from mevalonic acid 167 (Scheme 31). The first step of this synthesis is the conversion of the C-5 hydroxyl to the pyrophosphate via mevalonate and phosphomevalonate kinase, which affords pyrophosphate 168. The next step is a decarboxylation and elimination reaction performed in the presence of ATP which results in isopentyl pyrophosphate 170.185

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**Figure 26**: Fusidic acid binding to key amino acid contacts within EF-G.
The isopentyl pyrophosphate then isomerises before condensing twice with non-isomerised isopentyl phosphates forming 171 and 172. The last step involves coupling of two farnesyl pyrophosphates 172, which forms squalene 173.

Squalene is involved in the synthesis of multiple steroidal secondary metabolites such as cholesterol etc. The the first step in synthesis of fusidanes from squalene involves the epoxidation of one of the terminal alkenes affording 2,3-(S)-oxidosqualene 174. What follows is a complex tetracyclic ring formation, which forms via a cascade of reactions and includes the formation of 9 stereogenic centres. This is achieved via catalysis with oxidosqualene cyclase (OSC). OSCs are involved in the formation of over 100 different triterpene skeletons and in the biosynthesis of all of the fusidane family. In fact, the first point of diversity between the fusidanes is the identity of the OSC used in the catalysis of these cascade reactions. This process is known to form prosteryl cation intermediate 175, which then forms an alkene with the loss of a proton to generate 176 (Scheme 32).
Scheme 32: Biosynthesis of fusidic acid from squalene.

Tetracycle 176 is the last known intermediate in the biosynthesis of fusidic acid, but there is a number of steps left to perform. There is still the introduction of the carboxylic acid at C-21 and the two of hydroxyl groups (one acetylated) at C-11 and C-16 respectively and inversion of the stereochemistry at the C-3 alcohol. Finally, there is a loss of a methyl group at C-4.
Scheme 33: Enzymatic synthesis of fusidic acid intermediate 181.

Part of this synthesis has been achieved enzymatically by Mitsuguchi et al.\textsuperscript{191} This synthesis included the selective C-4 demethylation and inversion of the C-3 hydroxyl group. These researchers identified a gene cluster encoding for a number of enzymes. These enzymes convert 176 to 181 via the sequence given in Scheme 33. The sequence is initiated by oxidation of the C-3 hydroxyl group to ketone 177 and in turn the $\beta$-methyl group is oxidised in a stepwise fashion to alcoh 178 and thence to carboxylic acid 179. The carboxylic acid is then removed as CO$_2$ to form 180, before the ketone is reduced to form the C-3 hydroxyl with the correct configuration. To date, the remaining steps to form fusidic acid 166 from tetracycle 176 have not been explored, but it is reasonable to assume that the carboxylic acid and hydroxyl functional groups could be introduced regios- and stereo-selectively via further enzymatic transformations.
6.4 Total Synthesis of Fusidic acid

6.4.1 Formation of Tetracyclic Core

The first step towards the total synthesis of fusidic acid was published in 1972 by Dauben et al.\textsuperscript{192} The Dauben group concentrated on forming the tetracyclic decomposition product of fusidic acid 191.\textsuperscript{193} This synthesis was complicated by the need to form the \textit{trans-syn-trans} relative configuration of three cyclohexyl rings, which would correlate with fusidic acids core. These rings adopt a chair-boat-chair conformation which is an unusually constrained and its formation is energetically unfavourable under most reaction conditions.

\begin{center}
\textbf{Scheme 34:} Synthesis of tetracyclic fusidic acid decomposition product 191; (i) NaH, DMSO; (ii) KO-\textit{t-}Bu, Mel; (iii) H\textsubscript{2}O/\textit{AcOH}; (iv) triton B; (v) KOH, \textit{t-}BuOH, Mel; (vi) NaOH; (vii) (COCl)\textsubscript{2}; (viii) LiEt\textsubscript{2}Cu; (ix) \textit{mCPBA}; (x) BF\textsubscript{3}.Et\textsubscript{2}O; (xi) \textit{p-TsOH}; (xii) Li, NH\textsubscript{3}, \textit{t-BuOH}.
\end{center}

A previously synthesised bicyclic ketone 182\textsuperscript{194} was used as the starting material and was alkylated with bromo ketal ester 183 to form bicyclic ether 184. Bicyclic ether 184 was
then alkylated with methyl iodide before being hydrolysed with acetic acid to form a 1,5-diketone. This diketone was then cyclised to form the tricycle 185 using benzyltrimethylammonium hydroxide (Triton B). Tricyclic ketone 185 was selectively alkylated via application of base and methyl iodide to form 186. The carboxylic acid was then converted to an acid chloride using oxalyl chloride before being alkylated to the ethyl ketone using lithium diethylcuprate forming another 1,5-diketone 187. This 1,5-diketone was cyclised using triton B to form the third cyclohexane ring and hence the core tetracyclic structure of fusidic acid.

The cyclopentene ring of tetracycle 188 was then selectively epoxidised via using m-CPBA, to afford epoxide 189. The tert-butyl ether was cleaved using BF$_3$ diethyletherate, initiating a rearrangement reaction that delivered diketal 190 in a quantitative yield. The resulting C-15 hydroxyl was eliminated to give an intermediate enone. A dissolving metal reduction was used to reduce the enone to afford product 191.

This synthesis was achieved in a comparatively poor overall yield of 0.25%, although this was mostly due to the final two steps which were achieved with yields of 50% and 20% respectively. The Dauben group took a logical stepwise approach to the synthesis of the tetracyclic core, installing the correct stereochemistry of the C-4, C-8, C-10 and C-14 methyl groups in a decent yield (5.1%). The major problem was the conversion of the tetracyclic intermediate 188 to 191, which was achieved in a further 4 steps with only a 5% yield.

6.4.2 Conversion of 191 to a Known Fusidic Acid Degradation Product 193
The next step of the synthesis was published in 1982, also by Dauben et al.$^{195}$ The Dauben group attempted to find a synthetic pathway from the decomposition product 191 to 200, the known degradation product of fusidic acid. It was known that fusidic acid can be synthesised from degradation product 200 (Section 6.4.3).$^{196}$ The main difficulties facing this synthesis were the need to reduce the C-3 ketone stereoselectively and accomplish the stereoselective incorporation of two hydroxyl groups at C-11 and C-16.
Scheme 35: Synthesis of degradation product 200 from decomposition product 191: (i) (HOCH₂)₂, TsOH; (ii) LiAlH₄; (iii) DHP, TsOH; (iv) mCPBA; (v) Li, H₂NCH₂CH₂NH₂; (vi) CrO₃, Py; (vii) 80% aq AcOH; (viii) CrO₃, AcOH; (ix) H₂, PtO₂; (x) HOCH₂CH₂OH, TsOH; (xi) LiAlH₄; (xii) 80% aq AcOH; (xiii) NaOH; (xiv) CH₃OCH₂NEt₃⁺Cl⁻, reflux; (xv) LDA, (PhS)₂; (xvi) Pb(OAc)₄; (xvii) mCPBA; (xviii) Δ; (xix) H₂, Pd/C; (xx) TFA; (xxi) Ac₂O; AcOH, TsOH.

The first step in this part of the synthesis was the reduction of the C-17 ketone, which was achieved by selectively protecting the ketone at C-3 as an acetal (Scheme 35). The ketone at C-17 was then reduced using LiAlH₄ to afford a hydroxyl group which was then protected as a tetrahydropyranyl ether (THP) 192. The next step was the selective
epoxidation using mCPBA to form 193, before a reductive ring opening using lithium in ethylenediamine afforded 194 as a 5:3 mixture of diastereoisomers (9β-H:9α-H).

The C-11 hydroxyl was inverted via first oxidising the hydroxyl group with chromium trioxide forming a ketone at C-11. The acid labile protecting groups at C-3 and C-17 were removed before an exhaustive oxidation using chromium trioxide afforded 195. This newly formed trione was then selectively reduced to form the α-hydroxyl groups at C-3 and C-11. Selective reduction of the C-3 ketone was achieved by hydrogenation over PtO₂. The C-17 ketone was then protected as an acetal, before the C-11 ketone was subjected to a more aggressive reduction with LiAlH₄ affording the C-11 hydroxyl group with the correct configuration. This reduction appears to be due to steric hindrance on the α-face of the molecule resulting from the unusual configuration of the cyclohexane rings. This chair-boat-chair conformation appears to force the reducing agent to attack the β-face selectively.

The ketone was then deprotected with aqueous acetic acid, allowing for an epimerization reaction at C-13 using sodium hydroxide. This process afforded a ratio of 5:1 (13α-H:13β-H). The 13α-H product was isolated and purified before the C-3 and C-11 hydroxyl groups were protected as MOM ethers giving 197.

The next stage of the synthesis involved the introduction of the β-acetoxy group at C-16. The method used for this addition was the phenyl sulfonation at C-16 using LDA as a base, producing a mixture of isomers. The selective α-acetylation of C-16 was achieved using lead tetraacetate to afford sulfide 198. Sulfide 198 was then oxidised to a sulfoxide using mCPBA, before being heated, resulting in the elimination of the sulfoxide and the formation of an acetoxyenone. This compound was then selectively hydrogenated to form the C-16 β-acetoxy group of 199. The MOM groups were then removed and the hydroxyl groups were acetylated to give the degradation product 200. This part of the total synthesis was achieved in 21 steps in an overall yield of 0.45%.
6.4.3 Final Assembly of Fusidic Acid

The third and final stage of the total synthesis was published in 1977 by Tanabe et al\textsuperscript{196} (Scheme 36). The Tanabe group published their synthesis of fusidic acid from the known degradation product \textbf{200}, the total synthesis work on fusidic acid had been published. They hoped that it would provide an easier target for total synthesis by other research groups, an hypothesis which was eventually proved to be correct by Dauben \textit{et al}\textsuperscript{195} (Section 6.4.2). The Tanabe group achieved this by first forming degradation product \textbf{200} via the acetylation of the C-3 and C-11 hydroxyl groups of fusidic acid followed by the oxidative cleavage of the side chain using ruthenium tetroxide.

![Scheme 36: Synthesis of fusidic acid 166](image)

\textbf{Scheme 36:} Synthesis of fusidic acid 166; (i) LDA; (ii) Mel; (iii) Ac\textsubscript{2}O, Py; (iv) POCl\textsubscript{3}, Py; (v) unspecified selective saponification procedure.

6-Methyl-5-heptenoic acid \textbf{201} was prepared in a 50% yield from commercial precursors and was used as the eventual side chain.\textsuperscript{197} LDA (two equivalent) was then used to enolise the C-2 position of \textbf{201} and the anion was then condensed with the degradation product \textbf{200} to form a tetraol. The acid was then methylated with methyl iodide to afford \textbf{202}, before three of the four hydroxyl groups were selectively acetylated using acetic anhydride in pyridine to form triacetate \textbf{203}. The product was then dehydrated with phosphorous oxychloride to form the enoate \textbf{204}. Fusidic acid \textbf{166} is then isolated.
following an unspecified saponification procedure, which selectively deacetylated two of the three acetates. The Tanabe group neglected to include conditions or yield for the final step, but overall there were 5 steps with an overall yield of 6% from the first four. When combined with the Dauben group’s formation of the tetracyclic core, and their subsequent formation of the degradation product 200, the total synthesis of fusidic acid can be achieved in 38 steps, with an overall yield of less than 0.01%.

6.4.4 Formation of the Tetracyclic Core of Fusidic Acid via a Bicyclic Transannular Diels-Alder Reaction

Although there have been no further published methods for the total synthesis of fusidic acid, a different method for the formation of the unique trans-syn-trans tricyclohexane moiety was published by Jung et al.\textsuperscript{198} As shown in Section 6.4.1, the Dauben group formed the three cyclohexane rings using a stepwise approach, in which each ring was formed via aldol condensation reactions. This new approach involved a simultaneous formation of the tricyclic core via a bicyclic transannular Diels-Alder reaction (TADA). Previously, a similar trans-syn-trans tricyclohexane ring system 206 had been formed via a TADA reaction\textsuperscript{199} (Scheme 37).

![Scheme 37](image)

Scheme 37: Formation of trans-syn-trans-dodecahydrophenanthrene 206.\textsuperscript{199}

The Jung group used the chemistry described in Scheme 37 as a starting point in their search for an intermediate which could then be used to form fusidic acid analogues. The challenge faced with this approach was the stereochemistry of the published reaction was controlled by the C-3 methoxy group, C-11 ketone and the C-13 geminal diesters which could not be used in this case. Therefore, a new way of controlling the stereochemistry would need to be devised.
Scheme 38: Synthesis of bicyclic triene 212 from ethylester 207; (i) DIBAL, THF; (ii) TBSCl, Im, THF; (iii) HCC-Li, EDA/DMSO; (iv) t-BuLi, ClO₂Et; (v) Me₂CuLi, THF; (vi) DIBAL, THF; (vii) DMP, DCM; (viii) PMBO(CH₂)₃MgBr, THF; (ix) TBDPSCI, Im; (x) DDQ, DCM; (xi) CBr₄, PPh₃; (xii) MeCO₂CH₂PO(OCH₂CF₃)₂, NaH/DMF; (xiii) aq AcOH; (xiv) DMP, DCM; (xv) K₂CO₃; (xvi) DIBAL, TBAF; (xvii) (Cl₃CO)₂CO, Et₃N.

Bicyclic triene 212 was synthesised in 17 steps from enolate 207 in an overall yield of 15% (Scheme 38). The chemically interesting feature of 212 is the ether ‘bridge’ which links C-8 to C-12; this linkage helps to control the conformation of the bicyclic TADA reaction (Scheme 39).

Scheme 39: The conformation of dodecahedrons 211 and 212 during the bicyclic TADA reaction.
The bicyclic TADA reaction was then successfully performed on both 211 and 212 via heating to 110 °C, resulting in 213 and 214. However, when 211 was used, the resultant tricycle 213 had a cis-syn-cis conformation. When dodecahedron 212 with the ether ‘bridge’ was used, the TADA reaction afforded tricycle 214, which had a trans-syn-trans conformation. The alkene was then hydrogenated to form a closer fusidic acid homologue.

The bicyclic TADA reaction was highly successful with an excellent yield and complete stereocontrol. This process could, in theory, be used as a possible new route for the total synthesis of fusidic acid or its derivatives.

6.5 The Structure-Activity Relationship for the Antibiotic Activity of Fusidic Acid

In an attempt to ascertain the structure-activity relationship for the antibiotic activity of fusidic acid, a series of chemical modifications altering or removing these groups were performed by Godfrensen et al. (Figure 27). These modifications were tested with Staphylococcus aureus cells, which are known to be sensitive to fusidic acid.

![Figure 27](image)

**Figure 27**: The structure-activity relationship for the antibiotic activity of fusidic acid.
The chemistry performed by Godfretsen and the antibacterial assay results are surmised below.

Esterification of 166 gave the methyl ester which had an IC$_{50}$ value four orders of magnitude higher than fusidic acid, indicating that the acid functionality performs an essential role in binding.

The C-24 alkene was hydrogenated using a 5% palladium on calcium carbonate catalyst, leaving the enoate unaffected. This hydrogenated product had an antibacterial activity equivalent to fusidic acids, implying that the remote double bond has no significant role in binding to EF-G.

Complete hydrogenation of the alkenes resulted in an IC$_{50}$ value three orders of magnitude higher than fusidic acid. It was theorised that this reduction in activity was due to the change in the possible orientation of the carboxylic acid group. This theory was investigated via cyclopropanation of the C17-C20 electron deficient bond, which left the bond angle of the carboxylic acid group roughly equivalent to fusidic acid. The cyclopropyl derivative had a very similar activity to that of fusidic acid. This result indicates that it is the orientation of the carboxylic acid that is important in determining the antibiotic activity.

The C-16 acetyl group was removed using a concentrated sodium hydroxide solution. This resulting triol had an IC$_{50}$ value three orders of magnitude higher than fusidic acid. This implies the acetate group plays an active role in binding to EF-G, albeit a smaller role than the carboxylic acid group.

The C-3 hydroxyl group was selectively acetylated using $p$-TsOH, acetic acid and acetic anhydride. This diacetate had an IC$_{50}$ value three orders of magnitude larger than fusidic acid itself. Both hydroxyls could be acetylated using a mix of acetic anhydride and pyridine. Again, the IC$_{50}$ value increased again upon acetylation but by a much smaller
margin. These results indicate that the C-3 hydroxyl group plays an important role in binding, while the C-11 hydroxyl group plays a more marginal role. However, acetylation of these hydroxyl groups has less impact on the antibacterial activity of fusidic acid than esterification.
7.0 Fusidic Acid as a Splicing Inhibitor

7.1 Initial Splicing Data for Fusidic Acid

Like sordarin, fusidic acid was identified as a possible splicing inhibitor and was tested for pre-mRNA splicing inhibition in yeast cells. The initial results showed that not only is fusidic acid a splicing inhibitor, its activity is actually greater than both sordarin and sordaricin (Figure 28). In yeast cells, fusidic acid showed complete mRNA inhibition at a concentration of 2.5 mM, compared to sordarin, which showed only a partial inhibition of mRNA formation at 4 mM concentration. These results were somewhat surprising, considering that EF-2 appeared to be a closer homologue than EF-G due to the higher conservation of amino acid the contacts made with the sordaricin core.

![Figure 28](image-url)

**Figure 28:** [A] Splicing of ACT1 pre-mRNA for 20 mins in yeast whole cell extract with increasing amounts of fusidic acid in 4% DMSO. [B] Splicing of β-globin pre-mRNA for 2 hrs in HeLa nuclear extract with increasing amounts of fusidic acid in 1% DMSO.

This experiment was then repeated with human cells, and like sordarin, the splicing inhibition activity of fusidic acid increased when compared to yeast cells. In human cells almost complete inhibition was achieved at a concentration of 1 mM. This level of activity is roughly equivalent to the activity found for concentrations of 2.5 mM of
fusidic acid in yeast cells. This improvement in activity was expected, due to the conservation of Thr84 in the assumed binding site of EF-G in hSnu114, but not in yeast Snu114.

These initial results were very positive, as a second splicing inhibitor has been found which exhibits a greater activity than sordarin. It also has the advantage of being commercially available, which allowed us to work on derivatives of fusidic acid without requiring a lengthy biosynthesis.

7.2 Esterification of Fusidic Acid

The environment around the C-3 hydroxyl group of fusidic acid appears to be poorly conserved between EF-G and Snu114, implying that splicing inhibition could be improved via modification of this functional group.

This environment was explored via esterification of the C-3 hydroxyl with commercially available acids (Scheme 40). The first step involved the methylation of the carboxylic acid group using diazomethane. The C-3 hydroxyl group of the fusidic acid methyl ester was then esterified using a methodology described by Zander et al. This method involves reacting fusidic acid with a carboxylic acid using sulfonyl chloride resin and methyl imidazole as catalyst. This system was used because all reagents can be removed without the need for column chromatography. The sulfonyl chloride resin was removed via filtration, methyl imidazole was removed with Amberlyst-15 resin and excess acid was removed with polystyrene divinyl benzene amine scavenger resin.
Scheme 40: General method for the esterification of fusidic acid diazomethane and subsequently with carboxylic acids

7.2.1 Efficiency of Reaction Conditions

The initial conditions used by the Zander group involved mixing 1.2 equivalents of both acid and sulfonyl chloride resin, with 4 equivalents of methyl imidazole base at room temperature, in a dichloromethane solvent system. Using these conditions the reaction was tested using 2,2-dimethylpropanol 145 with both benzoic and hexanoic acids. Both of these reactions worked to a very high yield and excellent purity (Scheme 41).

Scheme 41: Test of esterification procedure

Fusidic acid 166 was then methylated to form methyl fusidic acid 218, before the esterification was attempted. The conditions previously detailed were then applied to fusidic acid with a number of different acids. Using these conditions only aliphatic acids were found to produce the desired esters (Scheme 42).
**Scheme 42**: Synthesis of cyclohexanoic ester 219; (i) TMSCH$_2$N$_2$, Tol/MeOH; (ii) Melm, Ps-SO$_2$Cl, C$_6$H$_5$CO$_2$H, DCM.

In an attempt to remedy this, revised conditions were attempted. These conditions involved heating the reaction to 50 °C in a closed system and increasing the quantities to 1.5 equivalents for both the carboxylic acid and sulfonyl chloride resin. These changes improved the efficiency of the reaction for aliphatic compounds and also resulted in the successful use of aromatic acids.

However, even with these new reaction conditions the chemistry was not universally successful. Electron deficient cinnamic acids were totally unreactive, despite the fact that cinnamic acid itself could be coupled in quantitative yield. No reaction was observed at all for any aliphatic α,β unsaturated acids and a very low yield for 2,2-dichloropropanoic acid and C-20 dodecanoic acid. Although the glycine residues 238 and 239 were unreactive, 237 could be coupled. The end Boc group is a useful functional group for further elaboration of the fusidic acid skeleton. This procedure allowed a focused library of fusidic acid ester derivatives to be synthesised quickly and efficiently without the need for further purification in most cases (Table 3).
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**Table 3:** The acids attempted in the esterification of fusidic acid (Conversions shown were assigned by $^1$H NMR).
7.2.2 The Regioselectivity Observed in the Esterification Reactions

These esterifications reactions are completely regioselective. These results can be justified by consideration of the conformation of fusidic acid.

Figure 29: Energy minimised structure of fusidic acid (hyperchem 8.0™)

The fusidic acid structure (Figure 29) demonstrates that there is significant steric hindrance between the C-11 hydroxyl and the methyl group at C-8 due to its 1,3-diaxial interactions. Axial substituents on six membered rings tend to react more slowly than when in an equatorial position. There is also appears to be significant steric hindrance from the substituents on C-1 (forming a “bay region” in the “left hand” cyclohexane ring) and the C-19 methyl group (Figure29). These features could all contribute to the regioselectivity of the esterification reactions.

Scheme 43: Proposed mechanism for esterification using sulfonyl chloride resin
The mechanism for this reaction may also provide an insight to this surprisingly high regioselectivity. In one of the proposed mechanisms (Scheme 43), the carboxylic acid effectively becomes a solid phase reagent before reacting with fusidic acid. This mechanism implies that the acid will have a much larger steric hinderance during the reaction than normally associated with esterification reaction using a sulfonyl chloride catalyst. This extra mass emphasises the inherent selectivity of the C-3 hydroxyl leading to the 100% regioselective esterification reactions.

7.2.3 Splicing Data for Fusidic Acid Ester Derivatives

The fusidic acid ester derivatives were tested for pre-mRNA splicing inhibition activity in yeast cells. The first fusidic acid derivative was formed when the carboxylic acid group was methylated, affording methyl ester 218 (Scheme 42). The removal of the acidic hydrogen led to a large increase in the observed levels of mRNA accumulation. This decrease in inhibition activity is similar to the effect observed when the carboxylic acid group of sordarin was methylated.

![Figure 30](image)

**Figure 30**: Splicing of ACT1 pre-mRNA for 20 min. in yeast whole cell extract with 4mM of indicated molecules at 4% DMSO final concentration.
All of the fusidic acid ester derivatives formed were methylated and as such the acidic protein is not present, so the base rate for comparing the splicing activity of these derivatives is the methyl ester fusidic acid derivative 218 (Figure 30). Most fusidic acid ester derivatives had an equivalent reduction in formed mRNA to that observed for the methyl ester fusidic acid derivative 218. This implies that the C-3 hydroxyl performs little to no function in the binding of fusidic acid to Snu114. Two ester derivatives did show an increased activity from the methyl fusidic acid base level. The first of these was the 4-chloro benzoic fusidic acid ester derivative 233, which showed a marked reduction in observed levels of both mRNA and splicing intermediates. The second of these was the trans-cinnamic fusidic acid ester derivative 229, which showed an almost complete inhibition of mRNA formation. This level of splicing inhibition is roughly equivalent to that observed in unmodified fusidic acid.

These results seem to imply that the C-3 hydroxyl is not significant to the binding of fusidic acid to Snu114. Therefore, it is a promising point of diversity to exploit when synthesising splicing inhibitors. The two most successful fusidic acid ester derivatives both had aromatic rings which were slightly electron deficient. The rigid planar nature of these side chains could also be important. These results could also imply a greater distance between the C-3 hydroxyl of fusidic acid and the binding sites in Snu114, than in EF-G.

7.3 Iodolactonization of Fusidic Acid
The side chain of fusidic acid has been replaced with a synthetic variation, by multiple research groups, with the intention of improving the antibacterial activity of fusidic acid. To date, these attempts involve replacing the current side chain with a similar, flexible one. There has not been an attempt to rigidify the side chain, which would afford a completely rigid structure.
This was achieved via an iodolactonization reaction, which involved using sodium hydrogen carbonate to deprotonate the carboxylic acid functional group and iodine to perform the lactonization reaction (Scheme 44). This reaction proceeded quickly and in a high yield producing three products, which were separated via HPLC. The major product was identified as cycloheptane 240, the two minor products are still unidentified but are believed to be 6-membered heterocyclic products. This result was unexpected as even 6-membered heterocyclic products are rare in iodolactonization reactions and are usually side products from 5-membered heterocyclic ring formations. The formation of 7-membered heterocycles appears to be completely novel for this type of reaction.

**Scheme 44**: Iodolactonization of fusidic acid.

**Scheme 45**: Mechanisms for general iodolactonization reactions and fusidic acid iodolactonization.
The generally accepted mechanism for 5-membered heterocycles forming in iodolactonization reactions is illustrated in Scheme 45. However, formation of the 7-membered heterocycle from fusidic acid side chain must occur in a slightly modified mechanism (Scheme 45). There appear to be a couple of contributing factors that could provide insight as to why this 7 membered heterocycle appears to be more favoured compared to other reactions of this type. This may be due to the fact that it is unusual to have an alkene group attached to the ring, let alone at the β-position to the acid. This planar 120° bond angle could dramatically alter which ring is favoured in the iodolactonization mechanism and could contribute to the 7-membered heterocycle being favoured.

Figure 31: Hyperchem optimised fusidic iodolactones; (A) α-lactone 157, energy = 38.5553 Kcal/mol, (B) β-lactone 157, energy = 40.3244, (C) lactone 158 (R)-C24, energy = 43.8549 Kcal/mol, (D) lactone 158 (S)-C24, energy = 44.5616 Kcal/mol.

Hyperchem analysis was performed on all four possible isomers, to calculate the ground state energies to ascertain the favoured isomers (Figure 31). However, these calculations imply that the favoured product would be the 6-membered iodolactones. Neither analysis of the mechanism or ground state energies has provided adequate insight, into the reasons why 7-membered iodolactones are favoured in this apparently unique case.
7.4 Protection Strategy for Side Chain Cleavage

Some of the other fusidic acid derivatives we wanted to produce required protection of the C-3/C-11 hydroxyls. This normally very well used and high yielding step proved to be more difficult than first imagined, in fact the C-3/C-11 hydroxyls seem to be remarkably unreactive. Chloroalkyl ether and several silyl agents were attempted without success, as well as all attempts to alkylate the hydroxyls with methyl iodide (Scheme 46).

![Scheme 46](image)

**Scheme 46:** Protection of hydroxyls and fusidic acid side chain cleavage; (i) TMSCH$_2$N$_2$, Toluene, MeOH; (ii) TBDMSCl, Imidazole, DMF; (iii) MOMCl, NaH, THF; (iv) MeI, K$_2$CO$_3$, MeOH; (v) Ac$_2$O, Pyridine.

To date, the only way that these hydroxyls have been successfully protected is via acetylation with acetic anhydride in pyridine. While this is fine for the protection of the hydroxyls there is however a problem with their eventual deprotection. The acetate group positioned at C-16 in fusidic acid is also likely to be removed via any attempt to deprotect the other two hydroxyl groups.
7.5 Side Chain Cleavage

As mentioned before, the side chain of fusidic acid is the only part of the molecule that has conformational flexibility and has possibly the most potential for improvement by modification. In fact, previous studies have shown that some of the more successful attempts at improving upon fusidic acid base level of antibacterial activity were via the modification of this side chain.

The first step in this modification is the cleavage of the side chain, the method chosen for this was oxidative cleavage with ozone\(^{200}\). To test this methodology unprotected fusidic acid was dissolved in a DMS/methanol mixture and ozone was bubbled through the solution. This reaction worked successfully in a high yield and good purity affording triketal 249 (Scheme 47).

![Scheme 47: Oxidative cleavage of fusidic acids side chain.](image)

So with this successful test, the hydroxyls were acetylated to protect them before the oxidative cleavage reaction. Unfortunately, the machine used for ozone production was no longer available and other methods for cleavage needed to be found. One of the methods attempted was via the use of ruthenium chloride and sodium periodate to oxidatively cleave the side chain.\(^{196}\) However, all attempts at this reagent method proved to be unsuccessful.
7.6 Regioselective Hydrogenation Reactions

The hydrogenation of fusidic acid was attempted as a method, with which, to investigate the structure-activity relationship of fusidic acids splicing inhibition activity. The C-24-ene group is associated with threonine-84 in EF-G and threonine-84 is retained in hSnu114 but not in yeast Snu114. Therefore, the removal of this functionality allows investigation into the possibility that the difference in splicing inhibition activity observed between human and yeast cells could be, at least partially, due to binding to the C-24-ene group.

Hydrogenation of the C-17-ene group would also provide interesting data. The double bond does not appear to directly bind to EF-2, but this bond does control the bond angle of carboxylic acid. Since the carboxylic acid group is key to fusidic acids splicing inhibition activity, it would be of great interest to see how this activity is affected by modifications to the bond angle.

The attempted hydrogenation of fusidic acid was performed using 5% palladium on carbon as a catalyst in a hydrogen atmosphere with an ethanol solvent system, affording 250 in an excellent yield (Scheme 48). The conditions used appear to be insufficient to hydrogenate the C-17 alkene so a new set of conditions were tried using platinum oxide in acetic acid, under a hydrogen atmosphere. Although these conditions have been used to successfully hydrogenate fusidic acid,200 they were unsuccessful here. This was probably due to much lower pressures of hydrogen obtained when compared to the literature procedure.

Scheme 48: Regioselective hydrogenation reactions.
7.7 Regioselective Epoxidation

An alternative investigation into the function of the C-24 alkene was attempted via an epoxidation reaction. The electron deficient nature of the C-24-C25 bond is the source of the binding of fusidic acid to threonine-84 in EF-G. Therefore, epoxidation of this alkene would result in a C-24-C-25 bond which is more electron deficient than an alkene group and could result in an overall improvement in splicing inhibition activity.

![Scheme 49: The regioselective epoxidation of the C-27 alkene.](image)

The reaction was performed initially using meta-chloroperbenzoic acid in dichloromethane, but without any stereoselective reagents to ascertain natural regioselectively (Scheme 49). This reaction proved to be completely regioselective, with epoxidation at the C-27 position successfully achieved in a good yield. The separation and isolation of both epoxidated products was achieved via HPLC reverse phase purification and the structure of the two compounds was confirmed by NMR spectroscopy and high resolution mass spectrometry.

7.8 Thiol Addition

One of the key aims of this project was to create a directed library of compounds and one way this could be achieved was via the introduction of a thiol group to fusidic acid. This would allow for the creation of a library of disulfide compounds quickly and easily.
Scheme 50: Introduction of thiol group via esterification and etherification; (i) Sulfonyl Chloride resin, MeIm/DCM; (ii) DMF, KH₂.

The first method attempted involved the esterification of the C-3 hydroxyl group using the same reagent system detailed in Section 7.2. This esterification was first performed with mercaptoacetic acid and methylated fusidic acid 221, which proved to be unsuccessful (Scheme 50). This lack of reactivity was probably due to some kind of interference from the thiol group. To circumvent this problem the thiol group was protected via acetylation, before the esterification reaction was retried using the same conditions. This time the reaction was successful and afforded thioacetate 255.

The second method attempted for thiol introduction was via alkylation with 1-bromo, 4-thioacetate but-2-ene, which was prepared from the nucleophilic substitution of potassium thioacetate to (E)-1,4-dibromobut-2-ene. Initially this reaction was tried using sodium hydride in tetrahydrofuran to deprotonate the hydroxyls, before (E)-S-4-bromobut-2-enyl ethanethioate was added. These reaction conditions proved insufficient and revised conditions were tried with potassium hydride in dimethyl formaldehyde. Unfortunately, these conditions also proved to be insufficient to cause alkylation. In Section 7.5 a protection method of the hydroxyl groups of fusidic acid was attempted using methyl iodide, which resulted in no reaction, and to date, there have been no reported C-3 or C-11 alkylation reactions. This is quite surprising considering the body of work published on producing fusidic acid derivatives. There appears to be some property to fusidic acids structure that is highly resistant to etherification at both hydroxyls.
7.9 Deacetylation and Lactonization of Fusidic Acid

The C-16 acetate group in fusidic acid binds to threonine-84 when bound to EF-G. Threonine-84 is conserved in hSnu114 but not in yeast Snu114, therefore could provide a reason for the apparent disparity between the splicing inhibition activities of fusidic acid in human and yeast cells.

![Scheme 51: De-acetylation and lactonization of fusidic acid; (i), NaOH; (ii) EtOH, reflux.](image)

The C-16 acetate group was removed via the application of concentrated sodium hydroxide solution (Scheme 51). This led to a 20% reduction in splicing inhibition activity, which implies the acetate group binds in a similar manner in Snu114 to that found in EF-G. An alternative explanation is that the newly formed hydroxyl interferes with the strong binding associated with the acid functionality.

In an attempt to investigate this, the acid was lactonised into a five membered heterocycle by refluxing the compound in ethanol. This removes both the hydroxyl and the acid functionality and when compared with methylated fusidic acid (which has the acid functionality blocked) allowed us to see the effect that the acetate group has in isolation. The splicing assay results for these compounds as well as an overall description of the overall structure-activity relationship, is detailed in Section 7.11.
7.10 The Structure-activity Relationship of Fusidic Acid as a Splicing Inhibitor

All the fusidic acid derivatives reported in Sections 7.3-7.9 were assayed for splicing inhibition, to ascertain the structure-activity relationship (Figure 32). These results were also compared to a previous study analysing the structure-activity relationship of fusidic acid as an antibiotic (Section 6.5). This comparison allows for the analysis of the strength of the homogeneity between the binding sites of fusidic acid in EF-G and Snu114.

The C24-25 bond of fusidic acid was modified via hydrogenation and epoxidation. Hydrogenation led to a 5% decrease in activity when compared to fusidic acid, while epoxidation of the C24-25 bond led to an equivalent activity. These results imply that the side chain plays a very small role in binding, which correlates to the results found by the Godfredsen group.\textsuperscript{200}

The carboxylic acid group has been modified in various ways, the simplest of which was via methylation. This fusidic acid methyl ester derivative had an activity 90% lower than that observed for fusidic acid. This implies that the carboxylic acid functional group is the main contributor to fusidic acid binding in Snu114. These results also correlate to the results found by the Godfredsen group and others.\textsuperscript{200,203,204} To date, all fusidic acid derivatives where the acidic proton has been removed have resulted in an antibacterial activity several orders of magnitude lower than fusidic acids.

![Figure 32: Details of the structure-activity relationship of fusidic acid as a splicing inhibitor.](image)
Fusidic acid was deacetylated, affording a hydroxyl at C-16, this led to a 25% reduction in splicing inhibition activity. In the x-ray structure of fusidic acid bound to EF-G\textsuperscript{205} both the acid and acetate groups appear to bind to threonine-84. Threonine-84 is conserved in Snu114, therefore cleavage of the acetate group resulting in a reduction in splicing inhibition activity is to be expected. However, the newly formed hydroxyl could also play a factor. In Section 7.9 the C-16 hydroxyl and carboxylic acid groups underwent a lactonization reaction affording a tetrahydro furan-2-one ring. This lactonization product also had a reduction in splicing inhibition activity of about 90% compared to fusidic acid. Although high temperatures are needed for lactonization to occur, this effect could lead to a reduction in binding between the carboxylic acid group and threonine-84.

The two hydroxyl groups of fusidic acid were acetylated, which led to a 20% reduction in splicing inhibition activity compared to fusidic acid. This implies that the two hydroxyls do play a small role in the binding to Snu114. In EF-G the C-3 hydroxyl binds to threonine-437, while the C-11 hydroxyl binds to both leucine-457 and glutamic acid-458. Leucine-457 is conserved in Snu114 and glutamic acid-458 is conservatively changed to aspartic acid, however threonine-437 is not conserved in Snu114. The loss of threonine-437 in Snu114 implies that the source of binding from the two hydroxyls is the C-11 hydroxyl. In Section 7.2 the C-3 hydroxyl of the fusidic acid methyl ester derivative \textsuperscript{221}, was regioselectively esterified using a number of different acids. Most of these esters resulted in equivalent activity to acid methyl ester derivative \textsuperscript{221}, however fusidic acid esters \textsuperscript{229} and \textsuperscript{233} did have significant splicing inhibition activity. Ester \textsuperscript{233} had an activity roughly 50% of that observed for fusidic acid, while ester \textsuperscript{229} had an activity equivalent to fusidic acid. The results for the C-3 hydroxyl do not correlate to the data obtained by the Godfredsen group, which indicated that the C-3 and C-11 hydroxyls both play significant parts in binding to EF-G. This lack of correlation implies that the environment around the C-3 hydroxyl of fusidic acid in Snu114 is significantly different to the equivalent environment in EF-G. However, it should be noted that the possibility exists that the activity observed for esters \textsuperscript{229} and \textsuperscript{233} could be due to fusidic acid binding somewhere else in the spliceosome.
7.11 Proposed Library Synthesis via Disulfide Coupling

In section 5.8, the use of disulfide coupling reactions as a method for producing libraries of sordarin derivatives was discussed. The same coupling methodology could also be used to produce fusidic acid derivative libraries. A thiol group has been successfully introduced to fusidic acid via the esterification of the C-3 hydroxyl with mercaptoacetic acid. The formation of disulfide libraries at the C-3 was chosen due to the findings of the structure-activity relationship study. The study found that the C-3 hydroxyl appears to play a minimal role in binding and thus far provided some of the more promising fusidic acid splicing inhibitors.

![Scheme 52: Proposed oxidative disulfide coupling of fusidic acid thiol derivative 260 with a variety of thiols.]

As in section 5.8, the suggested method for disulfide formation is via an oxidative coupling reaction using sodium iodide and hydrogen peroxide (Scheme 52). This method was chosen as the reagents will be removed during workup, eliminating the need for column chromatography purification and allowing for a smaller scale to be used.

7.12 Proposed Library Synthesis via Side Chain Replacement

The side chain of fusidic acid was successfully cleaved via an ozonolysis reaction which forms a ketone at C-17. The addition of synthetic side chains to the fusidic acid tetra cyclic core would be the logical next step, as the side chain appears to have little effect on binding. However, the carboxylic acid group would need to be conserved due to its importance in binding.
The attachment of a homogenous side chain has previously been performed by Tanabe et al. \(^{196}\) It was the Tanabe group’s intention to reproduce fusidic acid from the decomposition product 200. 6-methyl 5-heptenoic acid 201 was attached \textit{via} the application of butyl-lithium and diisopropylamine, affording tetra-ol 202. Tetra-ol 202 was then dehydrated to reform fusidic acid. Although the reproduction of fusidic acid is not our aim, the same method could be applied to form fusidic acid derivatives with varying side chains (Scheme 53).

\textbf{Scheme 53}: Side chain attachment performed by Tanabe \textit{et al.}\(^{196}\)

7.13 Future Snu114 Investigations

Thus far, various routes to produce synthetic sordarin and fusidic acid analogues have been explored. Through the use of these synthesised sordarin and fusidic acid derivatives it should be possible to determine a great deal about the role and structure of Snu114.

The first task would be to determine the point during spliceosome assembly and activation which these derivatives inhibit. Native gel electrophoresis can be used to analyse the formation of the E, A, B and C splicing complexes in both yeast and human \textit{in vitro} splicing systems.

The current splicing assay analyses the spliceosome throughout the splicing cycle. However, sordarin and fusidic acid blocks splicing before either of the transesterification reactions have taken place. Therefore, there is no known information on any possible
inhibition of the disassembly pathway. This pathway can be analysed by the use of yeast spliceosomes poised for disassembly. These affinity purified spliceosomes are assembled \textit{in vivo} and are associated with the disassembly factor Prp43.\textsuperscript{123} Prp43 purified spliceosomes have released the mRNA product but have not released the intron product or disassembled the snRNPs by U2/U6 unwinding. The addition of ATP to this purified spliceosomes induces spliceosome disassembly through the action of Brr2 (see Section 3.1). Therefore, spliceosome disassembly can be analysed by the addition of analogues to Prp43 purified spliceosomes before ATP.

A major step towards the analysis of the binding pocket would be the synthesis of fusidic acid and sordarin derivatives attached to both solid supports and photocrosslinking groups. The photocrosslinking groups would provide valuable binding information. The solid supported derivatives could be used to purify and identify proteins associated with analogues and assist in the purification of splicing complexes formed by the binding of fusidic acid and sordarin.

This would hopefully allow for isolation of the required quantities of Snu114 which could be used in high-throughput crystallization screens, which may lead to the discovery of conditions required for the crystallization of Snu114. Presently, there is no structural information on Snu114 and determining the structure of Snu114 will fill a gap in our knowledge of one of the core spliceosome proteins.
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**General Experimental**

$^1$H and $^{13}$H NMR spectra were recorded on a Varian Novo 300 MHz or a Bruker DPX 400 MHz spectrometers. Chemical shifts are given in parts per million (δ) and are referenced to tetramethylsilane and residual protonated solvent shifts. Standard abbreviations are used throughout (e.g. s = singlet; d = doublet, etc).

Infrared spectra were recorded using potassium bromide discs for thin film IR on a Mattson Genesis Series FTIR spectrometer or solid state on a Brucker Alpha-P series FTIR.

Low resolution mass spectra were recorded on a Micromass trio 2000 spectrometer for EI/CI spectra, and a Micromass Platform II spectrometer for electrospray spectra. All mass spectrometry results are reported in the form m/z.

Thin layer chromatography was performed using 0.25mm pre-coated aluminium-backed silica gel $60 \text{ F}_{254}$ plates and column chromatography with silica gel (particle size 40-63 µm), both supplied by Merck. Visualisation was achieved by; UV light, iodine, permanganate, vanillin or ceric sulphate.

Analytical and Semi prep HPLC was performed on Gilson multi module system with two model 306 pumps, an 805 manometric module, 811C dynamic mixer and a 118 UV detector.

Chiral HPLC was performed on a Hewett Packard 1050 series HPLC system.

Dry solvents were obtained by distilling from a suitable drying agent.

- DCM and chloroform were distilled from calcium hydride.
- THF was distilled from sodium with a benzophenone indicator
- Pyridine was purchased pre-dried
Cultivation of Sordarin

Experiment performed *Sordaria araneosa* (ATCC 36386) was cultivated on Emerson YSS agar (15.0 g soluble starch, 4.0 g yeast extract, 1.0 g K₂HPO₄, 0.5 g [MgSO₄·7H₂O], 20.0 g agar per litre distilled water) at 25 °C for 7 days. Frozen stocks were made by transferring actively growing mycelia to 15% (v/v) glycerol and storing at -85 °C.

The medium used for producing sordarin from *S. araneosa* was SR5-M6 as described by Tully, except that D-glucose and yeast extract were used instead of cerelose and Tastone-154, respectively. Pharmamedia was a kind gift from Trader's Protein (Tennessee, USA). FeSO₄·7H₂O and MgSO₄·7H₂O were added to the medium after autoclaving. The methods used to cultivate *S. araneosa* were generally based on those described by Tully. The contents of one vial containing thawed glycerol stock was added to 250 ml flasks containing 100 ml SR5-M6 and grown for 3 days at 26 °C and 300 rpm. One quarter of the flask was added to a 2000 ml flask containing 1000 ml SR5-M6 and grown for 10 days at 26 °C and 300 rpm. The mycelia were removed by filtration prior to sordarin purification.

Sordarin Extraction

Extraction method performed as described by Quingmei. Crude Sordarin broth material was received after filtration. Diaion HP-20 resin was added to the broth in a 20 g L⁻¹ ratio and stirred for 24 hrs. The resin was then packed into a column and the aqueous solution is collected. The sordarin is then extracted from the resin by flushing through with 1:1 acetone:water (250 ml) and acetone (200 ml). The acetone in the solvent mixture is then evaporated under vacuum and the sordarin is then extracted from the resulting aqueous phase using ethyl acetate (3 x 75 ml). The solvent is then evaporated under vacuum and the resulting oily resin was purified using standard column chromatography with a 1:3 hexane/ethyl acetate solvent system. This material was then further purified using HPLC. HPLC was performed using an Allsphere ODS 5μm 4.6x150 mm column, 0.5 ml/min flow rate and 150 μl injections of 1 g/ml of sample. Retention time for sordarin was 5.5 mins.
**In vitro pre-mRNA Splicing Assays**

Yeast pre-mRNA splicing extract was made from strain BJ2168 as described by Alvi et al.\textsuperscript{206} Splicing was carried out at 23 °C for 20 min, with 40% extract in 1X splicing buffer (60 mM potassium phosphate pH = 7.0, 3% PEG 8000, 2.5 mM MgCl$_2$, 2 mM ATP) and approximately 2 nM 32P labelled *ACT1* pre-mRNA. 10X stocks of molecules in 40% DMSO or 40% DMSO alone were added to reactions to give a final DMSO concentration of 4%. Splicing reactions were 10 µl and 4 µl was removed for RNA isolation from the reactions. RNA was then analyzed by 6% polyacrylamide/8 M urea gel electrophoresis.

Human pre-mRNA splicing was carried out with HeLa cell nuclear extract.\textsuperscript{207} Splicing was carried out at 30 °C for 2 hr, with 40% extract, 0.25 mM ATP, 10 mM phosphocreatine, 3.2 mM MgCl$_2$, 20 mM HEPES KOH pH = 7.9, 2% polyvinyl alcohol, 40 units RNAsin and approximately 2 nM 32P labelled β globin pre-mRNA. 10X stocks of molecules in 10% DMSO or 10% DMSO alone were added to reactions to give a final DMSO concentration of 1%. Splicing reactions were 10 µl and RNA was isolated from the whole reaction. RNA was then analyzed by 6% polyacrylamide/8 M urea gel electrophoresis.
Sordarin sample was provided by Sigma Aldrich as a sodium salt. Sordarin sodium salt 261 (5 mg, 9.7 μmol) was dissolved in a 1:1 water:DCM mix (2 mL), before 1M HCl (2-3 drops) was added to the mixture. The product was then extracted with DCM (3 x 1 mL) and dried with MgSO₄. The solvent was then evaporated to dryness in vacuo, affording sordarin 31 as a white powder (4.7 mg, 97.9% yield).

Rᵣ = 0.4 (6:4:0.1 ethyl acetate:hexane:acetic acid); ¹H NMR (CDCl₃, 400 Hz) δ = 0.72 (d, 3H, J = 6.7 Hz, 1), 0.90 (d, 3H, J = 6.8 Hz, 17), 0.96 (d, 3H, J = 6.8 Hz, 18), 1.08-1.16 (m, 2H, 4a, 3a), 1.17-1.21 (m, 2H, 14a, 7a) 1.23 (d, 3H, J = 6.3 Hz, 26), 1.60-2.10 (m, 5H, 2, 3b, 4b, 7b, 14b), 2.24-2.28 (m, 1H, 16), 2.62 (t, 1H, J = 3.9 Hz, 9), 3.14 (dd, 1H, J = 9.2, 2.8 Hz, 24), 3.35 (s, 3H, OMe), 3.62-3.68 (m, 2H, 15a, 25), 3.82 (d, 1H, J = 3.8 Hz, 22), 4.01 (d, 1H, J = 9.2 Hz, 15b), 4.14 (t, 1H, J = 3.5 Hz, 23), 4.60 (bs, 1H, 21), 6.00 (d, 1H, J = 3.3 Hz, 10), 9.67 (s, 1H, 19); ¹³C NMR (CDCl₃, 400 Hz) δ = 16.4 (CH₃, 1), 17.1 (CH₃, 26), 20.2 (CH₃, 18), 21.6 (CH₃, 17), 25.2 (CH₂, 4), 26.6 (CH, 16), 28.2 (CH₂, 7), 28.3 (CH₂, 14), 30.0 (CH, 2), 31.0 (CH₂, 3), 40.3 (CH, 6), 40.8 (CH, 5), 45.3 (CH, 9), 56.6 (CH₃, OMe), 64.7 (C, 13), 66.0 (C, 8), 67.6 (CH, 25), 69.2 (CH, 22), 71.4 (C, 12), 73.3 (CH₂, 15), 78.8 (CH, 24), 97.2 (CH, 21), 129.8 (CH, 10), 147.4 (C, 11), 173.8 (C, 20), 204.0 (CH, 19); LR-MS ES-[M-H]⁻ = 491 (100%).
Sordarin-1-methylester; 1,4-Methano-s-indacene-3a(1H)-carboxylic acid, 8a-[(6-deoxy-4-O-methyl-β-D-altropyranosyl)oxy][methyl]-4-formyl-4,4a,5,6,7a,8,8a-octahydro-7-methyl-3-(1-methylethyl)-methyl ester, (1R,3aR,4S,4aR,7R,7aR,8aS)- (131)

![Chemical structure](image)

Sordarin 31 (4.5 mg, 9.1 µmol) was dissolved in 2:1 toluene:methanol mix (0.3 mL), before the solution was then placed under an inert N₂ atmosphere and cooled to 0 °C. Trimethylsilyl diazomethane (30 µL, 0.06 mmol) was then injected into the solution and the reaction was allowed to warm to room temperature and left stirring for 8 hrs. The reaction was then quenched using 10% acetic acid solution, extracted with ethyl acetate (3 x 1.5 mL) and dried with MgSO₄. The solution was then evaporated to dryness in vacuo, affording 131 as a white powder (4.5 mg, yield = 98.0%).

**Rf** = 0.55 (60:40:1 (ethyl acetate:hexane:acetic acid); **¹H NMR** (CDCl₃, 400 Hz) δ = 0.69 (d, 3H, J = 6.5 Hz, 1), 0.82-0.92 (m, 1H, 4a), 0.90 (d, 3H, J = 6.7 Hz, 17), 0.96 (d, 3H, J = 6.7 Hz, 18), 1.04-1.20 (m, 4H, 7a, 14a, 14b, 3a), 1.23 (d, 3H, J = 6.3 Hz, 26), 1.47-2.07 (m, 6H, 4b, 7b, 3b, 2, 5, 6), 2.10-2.25 (m, 1H, 16), 2.67 (t, 1H, J = 3.8 Hz, 9), 3.14 (dd, 1H, J = 3.1, 9.2 Hz, 24), 3.35 (s, 3H, OMe), 3.56-3.68 (m, 2H, 15a, 25), 3.70 (s, 3H, CO₂Me), 3.81 (d, 1H, J = 4.1 Hz, 22), 3.87 (d, 1H, J = 9.4 Hz, 15b), 4.14 (t, 1H, J = 3.4 Hz, 23), 4.57 (bs, 1H, 21), 5.98 (d, 1H, J = 3.3 Hz, 10), 9.65 (s, 1H, 19); **¹³C NMR** (CDCl₃, 300 Hz) δ = 17.3 (CH₃, 1), 18.1 (CH₃, 26), 21.0 (CH₃, 18), 22.2 (CH₂, 17), 26.4 (CH₂, 4), 27.6 (CH, 16), 29.0 (CH₂, 7), 29.3 (CH₂, 14), 31.0 (CH, 2), 32.0 (CH₂, 3), 41.3 (CH, 6), 41.4 (CH, 5), 46.1 (CH, 9), 51.8 (CH₃, CO₂Me), 57.5 (CH₃, OMe), 58.9 (C, 13), 65.7 (C, 8), 66.9 (CH, 23), 68.3 (CH, 25), 70.3 (CH, 22), 72.0 (C, 12), 74.5 (CH₂, 15),...
79.9 (CH, 24), 98.1 (CH, 21), 130.5 (CH, 10), 148.2 (C, 11), 172.6 (C, 21), 204.1 (CH, 19); **LR-MS**: ES+ [M+Na]^+ 529 (100%); [M+H]^+ 507 (12%); **HR-MS**: ES+ calc for C_{29}H_{42}O_{8}Na 529.2772, found 529.2775.

Sordaricin (32)

Sordarin (4.7 mg, 9.5 mmol) was dissolved in ethanol (0.4 mL), before HCl (10M, 64 µL) was added to the mixture. The solution was stirred for 1 hr before being mixed with H_{2}O (5 mL), extracted with EtOAc (3 x 5 mL) and dried with MgSO_{4}. The solvent was then evaporated to dryness **in vacuo** resulting in sordaricin 32 as a white powder. (3.0 mg, 94.7% yield).

\[ ^{1}H \text{ NMR} (\text{CDCl}_3, 400 \text{ Hz}) \delta = 0.76 (3H, d, J = 6.9 \text{ Hz}, 1), 0.78-0.90 (m, 1H, 4a), 0.92 (3H, d, J = 6.8 \text{ Hz}, 17), 0.97 (3H, d, J = 6.8 \text{ Hz}, 18), 1.12-1.15 (m, 3H, 3a, 3b, 14a), 1.56 (1H, dd, J = 14.5, 6.3 \text{ Hz}, 7a), 1.74-2.10 (m, 6H, 4b, 7b, 14b, 6, 5, 2), 2.24-2.34 (m, 1H, 16), 2.36 (1H, bt, J = 3.9 \text{ Hz}, 9), 3.39 (1H, d, J = 11.1 \text{ Hz}, 15a), 4.07 (1H, d, J = 11.1 \text{ Hz}, 15b), 6.00 (1H, dd, J = 3.4, 1.2 \text{ Hz}, 24), 9.69 (1H, s, 19); ^{13}C \text{ NMR} (\text{CDCl}_3, 300 \text{ Hz}) \delta = 17.6 (\text{CH}_3, 1), 21.3 (\text{CH}_3, 18), 22.8 (\text{CH}_3, 17), 26.4 (\text{CH}_2, 4), 27.8 (\text{CH}, 16), 28.1 (\text{CH}_2, 7), 30.0 (\text{CH}_2, 14), 31.3 (\text{CH}, 2), 32.2 (\text{CH}_2, 3), 41.3 (\text{CH}, 6), 42.2 (\text{CH}, 5), 47.5 (\text{CH}, 9), 59.5 (\text{C}, 13), 66.7 (\text{C}, 8), 67.0 (\text{CH}_2, 15), 77.5 (\text{C}, 12), 131.0 (\text{CH}, 10), 148.7 (\text{C}, 11), 171.9 (\text{C}, 20), 206.0 (\text{CH}, 19); **LR-MS**: ES+ [M+Na]^+ 355 (100%); [M+H]^+ 333 (13%); ES-[M-H]^:-331 (100%); **HR-MS**: ES+ calc for C_{20}H_{29}O_{4} 333.2061, found 333.2061.
**General Procedure for Acetylation of Monosaccharides**

Reaction performed as described by Chaterjee et al.\textsuperscript{209} The monosaccharide (1.5 g) and Sodium acetate (1 eq) were dissolved in acetic anhydride (12.5 mL, 10 mmol). The solution was heated to 100 °C for 4 hrs. The product was allowed to cool to room temperature and then shaken with iced water. The acetylated monosaccharide product proceeded to precipitate out of solution, which was then filtered and evaporated to dryness \textit{in vacuo}.

**General Procedure for Bromination of Acetylated Monosaccharides**

Reaction was performed as described by Sznaidman \textit{et al.}\textsuperscript{210} The acetylated monosaccharide (1.5 g) was dissolved in dichloromethane (25 mL). The solution was cooled to 0 °C before 33% HBr in AcOH (1 eq) was added. The solution was then allowed to warm to room temperature and stirred for 3 hrs. A further aliquot of 33% HBr in AcOH (3 eq) was added and the solution was stirred for another hour. The solution was diluted with dichloromethane, washed with NaHCO\textsubscript{3}, dried with MgSO\textsubscript{4}, and then evaporated to dryness \textit{in vacuo}.

**General Procedure for the Formation of Glycals**

Reaction performed as described by Oberthur \textit{et al.}\textsuperscript{211} The 1-bromo acetylated monosaccharide (0.5 g), 1-methylimidazole (1 eq) and Zinc dust (6 eq) were mixed in ethyl acetate (7 mL). The mixture was then heated to reflux for 45 minutes, before being allowed to cool to room temperature. The mixture was then filtered through Celite, washed with 1M HCl (7 mL), saturated sodium hydrogen carbonate solution (7 mL), and dried with MgSO\textsubscript{4}. The solution was then evaporated to dryness \textit{in vacuo}. Crude product was purified by flash column chromatography (hexane:ethyl acetate 3:1).
**D-Glucose pentaacetate (133)**

![D-Glucose pentaacetate reaction](image)

D-Glucose 132 (1.8 g, 10 mmol) was reacted using conditions detailed in the general procedure for acetylation of monosaccharides, affording D-glucose pentaacetate 133 (3.01 g, yield = 96%) as a white solid. D-Glucose pentaacetate 133 was characterised as containing a 4:1 mixture of anomers. $^{13}$C peaks listed are for the major β-anomer.$^{212}$

**Rf:** 0.65 (1:1 ethyl acetate:hexane); $^1$H NMR: (CDCl$_3$, 300 Hz), δ = 2.02 (s, 3H, OAc), 2.04 (s, 6H, OAc), 2.10 (s, 3H, OAc), 2.12 (s, 3H, OAc), 3.85 (ddd, 0.8H, $J$ = 9.9, 4.4, 2.1 Hz, 5β), 4.06-4.17 (m, 1.2H, 6αα, 5αα, 6αβ), 4.22-4.34 (m, 1H, 6βα, 6ββ), 5.05-5.19 (m, 2H, 2α, 2β, 4α, 4β), 5.26 (t, 0.8H, $J$ = 9.4 Hz 3β), 5.48 (t, $J$ = 9.9 Hz, 0.2H, 3α), 5.72 (d, $J$ = 8.2 Hz, 0.8H, 1β), 6.34 (d, $J$ = 3.6 Hz, 0.2H, 1α); $^{13}$C NMR: (CDCl$_3$, 300Hz), δ = 20.6 (CH$_3$, OAc), 20.6 (CH$_3$, OAc), 20.7 (CH$_3$, OAc), 20.7 (CH$_3$, OAc), 20.9 (CH$_3$, OAc), 61.5 (CH$_2$, 6), 67.8 (CH, 4), 70.3 (CH, 2), 72.7 (CH, 5), 72.8 (CH, 3), 91.7 (CH, 1), 169.0 (C=O, OAc), 169.2 (C=O, OAc), 169.4 (C=O, OAc), 170.1 (C=O, OAc), 170.6 (C=O, OAc); LR-MS: ES+ [M+Na]$^+$ = 413 (100%).

**2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (134)**

![2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide reaction](image)

D-Glucose pentaacetate 133 (3.60 g, 9.23 mmol) was reacted using conditions detailed in the general procedure for bromination of acetylated monosaccharides, affording 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide 134 (3.79 g, yield = 96.5%) as a yellow oil. 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide 134 was characterised as containing solely the α-anomer.$^{213}$
\(^1\)H NMR: (CDCl\(_3\) 300 Hz); \(\delta = 1.97\) (s, 3H, OAc), 1.99 (s, 3H, OAc), 2x2.04 (s, 6H, OAc), 3.92-4.10 (m, 1H, 6a), 4.14-4.31 (m, 2H, 6b, 5), 4.77 (dd, \(J = 10.2\), 4.0 Hz, 2), 5.10 (t, 1H, \(J = 9.8\) Hz, 4), 5.49 (t, 1H, \(J = 9.8\) Hz, 3), 6.54 (d, \(J = 4.0\) Hz, 1); \(^{13}\)C NMR: (CDCl\(_3\) 300 Hz), \(\delta = 20.5\) (CH\(_3\), OAc), 20.6 (CH\(_3\), OAc), 20.7 (CH\(_3\), OAc), 20.8 (CH\(_3\), OAc), 61.0 (CH\(_2\), 6), 67.2 (CH, 4), 70.2 (CH, 3), 70.6 (CH, 2), 72.2 (CH, 5), 86.6 (CH, 1), 169.5 (C=O, OAc), 169.8 (C=O, OAc), 169.9 (C=O, OAc), 170.5 (C=O, OAc); LR-MS: ES\(^+\) [M+Na]\(^+\) = 434 (80%), 436 (80%).

3,4,6-Tri-O-acetyl-D-glucal (135)

2,3,4,6-Tetra-O-acetyl-\(\alpha\)-D-glucopyranosyl bromide 134 (0.50 g, 1.2 mmol) was reacted using conditions detailed in general glycal formation procedure. This reaction afforded a crude brown oil, which was purified by flash column chromatography (hexane:ethyl acetate 3:1) affording 3,4,6-tri-O-acetyl-D-glucal 135 (0.12 g, yield = 36.2%) as a pale yellow oil.\(^{214}\)

Rf: 0.76 (1:1 ethyl acetate:hexane); \(^1\)H NMR: (CDCl\(_3\) 300 Hz), \(\delta = 1.98\) (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.03 (s, 3H, OAc), 4.02-4.26 (m, 2H, 5, 6a), 4.33 (dd, 1H, \(J = 11.8\), 5.5 Hz, 6b), 4.78 (dd, 1H, \(J = 6.1\), 3.2 Hz, 2), 5.15 (dd, \(J = 7.5\), 5.8 Hz, 4), 5.23-5.31 (m, 1H, 3), 6.41 (dd, 1H, \(J = 6.1\), 1.3 Hz, 1); \(^{13}\)C NMR: (CDCl\(_3\) 300 Hz), \(\delta = 20.7\) (CH\(_3\), OAc), 20.8 (CH\(_3\), OAc), 21.0 (CH\(_3\), OAc), 61.4 (CH, 6), 67.2 (CH, 4), 67.4 (CH, 3), 74.0 (CH, 5), 99.0 (CH, 2), 145.6 (CH, 1), 169.6 (C=O, OAc), 170.4 (C=O, OAc), 170.6 (C=O, OAc); LR-MS: ES\(^+\) [M+Na]\(^+\) = 295 (100%).
**β-D-Galactose 1,2,3,4,6-pentaacetate (264)**

β-D-Galactose 263 (1.05 g, 5.6 mmol) was reacted using conditions detailed in general procedure for the acetylation of monosaccharides, affording β-D-Galactose 1,2,3,4,6-pentaacetate 264 (1.98 g, yield = 93.5%) as a white powder. NMR spectroscopy showed that the product obtained was purely the β-anomer.\textsuperscript{215}

**\textsuperscript{1}H NMR:** (CDCl\textsubscript{3}, 300 Hz), δ = 2.00 (s, 3H, OAc), 2.05 (bs, 6H, 2xOAc), 2.13 (s, 3H, OAc), 2.17 (s, 3H, OAc), 3.98–4.12 (m, 3H, 5, 6a, 6b), 5.10 (dd, J = 10.4, 3.5 Hz, 3), 5.33 (dd, 1H, J = 10.4, 8.3 Hz, 2), 5.44 (dd, 1H, J = 3.5, 0.8 Hz, 4), 5.69 (d, J = 8.3 Hz, 1); \textsuperscript{13}C NMR: (CDCl\textsubscript{3}, 300 Hz), δ = 20.5 (CH\textsubscript{3}, OAc), 20.6 (CH\textsubscript{3}, OAc), 20.6 (CH\textsubscript{3}, OAc), 20.8 (CH\textsubscript{3}, OAc), 22.2 (CH\textsubscript{3}, OAc), 61.0 (CH\textsubscript{2}, 6), 66.8 (CH, 4), 67.9 (CH, 2), 70.8 (CH, 3), 71.7 (CH, 5), 92.2 (CH, 1), 169.0 (C=O, OAc), 169.4 (C=O, OAc), 170.0 (C=O, OAc), 170.1 (C=O, OAc), 170.4 (C=O, OAc); LR-MS: ES\textsuperscript{+} [M+Na]\textsuperscript{+} = 413 (100%).

**2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide (265)**

β-D-Galactose 1,2,3,4,6-pentaacetate 264 (1.07 g, 2.7 mmol) was reacted using conditions detailed in the general procedure for bromination of acetylated monosaccharides, affording 2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide 265 (0.73 g, yield = 65.7%) as a yellow oil. NMR spectroscopy showed that the product obtained was purely the α-anomer.\textsuperscript{215}
\[ ^1H \text{NMR: (CDCl}_3, 300 \text{ Hz}, \delta = 2.02 \text{ (s, 3H, OAc, 2.07 (s, 3H, OAc, 2.13 (s, 3H, OAc, 2.16 (s, 3H, OAc, 4.09 (dd, 1H, } J = 11.4, 6.3 \text{ Hz, 6a), 4.16 (dd, 1H, } J = 11.4, 6.8 \text{ Hz, 6b, 4.42-4.50 (m, 1H, 5), 5.06 (dd, 1H, } J = 10.6, 4.0 \text{ Hz, 2), 5.37 (dd, 1H, } J = 3.3, 10.6 \text{ Hz, 3), 5.47 (dd, 1H, } J = 3.3, 1.1 \text{ Hz, 4), 6.71 (d, 1H, } J = 4.0 \text{ Hz, 1); } ^13\text{C NMR: (CDCl}_3, 300 \text{ Hz),} \]
\[ \delta = 20.5 \text{ (CH}_3, \text{ OAc), 20.6 \text{ (CH}_3, \text{ OAc), 20.7 \text{ (CH}_3, \text{ OAc), 66.8 \text{ (CH}_2, 6), 67.0 \text{ (CH, 4), 67.8 \text{ (CH, 2), 68.0 (CH, 3), 71.1 (CH, 5), 88.2 (CH, 1), 169.7 (C=O, OAc), 169.9 (C=O, OAc), 170.0 (C=O, OAc), 170.3 (C=O, OAc); LR-MS: ES+ [M+Na]^+ = 434 (80\%), 436 (80\%).} \]

3,4,6-Tri-\textit{O}-acetyl-\textit{D}-galactal (266)

\[ \text{AcO}_2\text{Br} \xrightarrow{\text{Zn/CuSO}_4} \]

1-Bromo \textit{D}-galactose butaacetate 265 (0.45 g, 1.09 mmol) was reacted using conditions detailed in general acetylation of monosaccharides procedure. This reaction afforded galactal 3,4,6-triacetate 266 (0.24 g, yield = 78.8\%) as a colourless oil.\[ ^1H \text{NMR: (CDCl}_3, 400 \text{ Hz), } \delta = 1.96 \text{ (s, 3H, OAc), 2.01 \text{ (s, 3H, OAc, 2.06 \text{ (s, 3H, OAc, 4.10-4.16 \text{ (m, 2H, 6a, 6b), 4.21-4.29 \text{ (m, 1H, 5), 4.65 \text{ (dd, 1H, } J = 6.3, 2.6, 1.5 \text{ Hz, 2), 5.34-5.36 \text{ (m, 1H, 4), 5.45-5.51 \text{ (m, 1H, 3), 6.40 (dd, 1H, } J = 6.3, 1.7 \text{ Hz, 1); } ^13\text{C NMR: (CDCl}_3, 400\text{Hz), } \delta = 20.5 \text{ (CH}_3, \text{ OAc), 20.6 \text{ (CH}_3, \text{ OAc), 20.7 \text{ (CH}_3, \text{ OAc), 61.9 \text{ (CH}_2, 6), 67.8 \text{ (CH, 4), 72.7 \text{ (CH, 5), 90.4 (CH, 3), 98.8 (CH, 2), 145.3 (CH, 1), 170.1 (C=O, OAc), 170.2 (C=O, OAc), 170.5 (C=O, OAc); LR-MS: ES+ [M+Na]^+ = 295 (100%).} \]

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D-Arabinopyranose-1,2,3,4-tetraacetate (268)

D-Arabinose 267 (1.06 g, 6.7 mmol) was reacted using conditions detailed in general acetylation of monosaccharides procedure. The reaction afforded α-D-arabinopyranose, 1,2,3,4-tetraacetate 268 (1.98 g, yield = 93.5%) as a thick yellow oil. NMR spectroscopy showed that the product obtained was purely the β-anomer.\(^\text{217}\)

\(^{1}\text{H NMR:}\) (CDCl\(_3\), 300 Hz), δ = 2.01 (s, 3H, OAc), 2.03 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.11 (s, 3H, OAc), 3.76 (dd, 1H, J = 13.0, 1.9 Hz, 5a), 4.01 (dd, 1H, J = 13.0, 3.7 Hz, 5b), 5.09 (dd, J = 9.1, 3.5 Hz, 3), 5.23-5.26 (m, 2H, 2, 4), 5.61 (d, 1H, J = 6.9 Hz, 1); \(^{13}\text{C NMR:}\) (CDCl\(_3\), 300 Hz), δ = 20.6 (CH\(_3\), OAc), 20.6 (CH\(_3\), OAc), 20.7 (CH\(_3\), OAc), 20.8 (CH\(_3\), OAc), 63.8 (CH\(_2\), 5), 67.2 (CH, 4), 68.1 (CH, 2), 69.8 (CH, 3), 92.1 (CH, 1), 169.0 (C=O, OAc), 169.3 (C=O, OAc), 169.9 (C=O, OAc), 170.1 (C=O, OAc); LR-MS: ES+ [M+Na]** = 341 (100%).

2,3,4-Tri-O-acetyl-α-D-arabinopyranosyl bromide (269)

β-D-Arabinopyranose butaacetate 268 (1.06 g, 6.7 mmol) was reacted using conditions detailed in the general bromination procedure. This reaction afforded 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl bromide 269 (1.60 g, yield = 73.8%) as a yellow oil. NMR spectroscopy showed that the product obtained was purely the α-anomer.\(^\text{218}\)
$^{1}$H NMR: (CDCl$_3$, 300 Hz), $\delta$ = 2.00 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.13 (s, 3H, OAc), 3.92 (dd, 1H, $J = 13.4$, 1.7 Hz, 5a), 4.19 (d, 1H, $J = 13.4$ Hz, 5b), 5.06 (ddd, 1H, $J = 11.6$, 3.8, 1.4 Hz, 2), 5.27-5.41 (m, 2H, 3, 4), 6.67 (d, 1H, $J = 3.9$ Hz, 1); $^{13}$C NMR: (CDCl$_3$, 300 Hz), $\delta$ = 20.6 (s, 3H, OAc), 20.7 (s, 3H, OAc), 20.8 (s, 3H, OAc), 64.7 (CH$_2$, 5), 67.6 (CH, 3), 67.9 (CH, 2), 68.0 (CH, 4), 89.7 (CH, 1), 170.1 (C=O, OAc), 170.1 (C=O, OAc), 169.8 (C=O, OAc); LR-MS: ES$^+$ [M+Na]$^+$ = 362 (100%), 364 (100%).

3,4-Diacetyl-\(D\)-arabinal (270)

\[
\begin{array}{c}
\text{AcO} \quad \text{OAc} \\
\text{OAc} \quad \text{CuSO}_4/Zn \\
\text{Br}^+ \\
269 \\
\text{AcO} \quad \text{OAc} \\
\text{OAc} \\
270
\end{array}
\]

2,3,4-Tri-\(O\)-acetyl-\(\alpha\)-\(D\)-arabinopyranosyl bromide 269 (0.25 g, 0.75 mmol) was reacted using conditions detailed in the general glycal formation procedure. This reaction afforded 3,4-diacetyl-\(D\)-arabinal 270 (0.14 g, yield = 95.2%) being isolated as a colourless oil.$^{218}$

$^{1}$H NMR: (CDCl$_3$, 400 Hz), $\delta$ = 2.00 (s, 3H, OAc), 2.01 (s, 3H, OAc), 3.87-3.94 (m, 2H, 5a, 5b), 4.78 (dd, 1H, $J = 6.0$, 5.1 Hz, 2), 5.05-5.25 (m, 1H, 4), 5.35 (m, 1H, 3), 6.44 (dd, 1H, $J = 6.0$, 0.5 Hz, 1); $^{13}$C NMR: (CDCl$_3$, 400 Hz), $\delta$ = 20.8 (CH$_3$, OAc), 21.0 (CH$_3$, OAc), 62.8 (CH$_2$, 5), 62.9 (CH, 3), 65.9 (CH, 4), 97.5 (CH, 2), 147.8 (CH, 1), 169.9 (C=O, OAc), 170.5 (C=O, OAc); LR-MS: ES$^+$ [M+Na]$^+$ = 223 (70%).

\(D\)-Galactopyranose, 6-deoxy, 1,2,3,4-tetracetate (272)

\[
\begin{array}{c}
\text{HO}_{\text{OH}} \quad \text{OH} \\
\text{AcO}_{\text{Ac}} \quad \text{NaOAc} \\
271 \\
\text{AcO}_{\text{Ac}} \quad \text{OAc} \\
\text{OAc} \\
272
\end{array}
\]

\(D\)-Fucose 271 (0.25 g, 1.5 mmol) was reacted using conditions detailed in the general procedure for acetylation of monosaccharides, affording \(D\)-galactopyranose, 6-deoxy,
1,2,3,4-tetracetate 272 as a thick yellow oil (0.50 g, yield = 97.7%). NMR spectroscopy showed that the product obtained was 4:1, β:α anomer mix.

\[ ^1H \text{ NMR: (CDCl}_3, 400 \text{ Hz), } \delta = 1.09 (d, 0.6H, J = 6.5 \text{ Hz, } 6\alpha), 1.22 (d, 2.4 \text{ H, } J = 6.5 \text{ Hz, } 6\beta), 1.94 (s, 0.6H, OAc } \alpha), 1.95 (s, 0.6H, OAc } \alpha), 2.08 (s, 0.6H, OAc } \alpha), 2.11 (s, 0.6H, OAc } \alpha), 1.93 (s, 2.4H, OAc } \beta), 1.97 (s, 2.4H, OAc } \beta), 2.05 (s, 2.4H, OAc } \beta), 2.12 (s, 2.4H, OAc } \beta), 3.89 (dq, 0.8H, J = 6.5, 1.0 \text{ Hz, } 5\beta), 4.21 (q, 0.2H, J = 6.4 \text{ Hz, } 5\alpha), 5.01 (dd, 0.8H, J = 10.4, 3.4 \text{ Hz, } 3\beta), 5.21 (dd, J = 3.4, 1.0 \text{ Hz, } 0.8H, 4\beta), 5.25-5.28 (m, 1.6H, 2\alpha, 3\alpha, 4\alpha and 2\beta), 5.62 (d, 0.8H, J = 8.3 \text{ Hz, } 1\beta), 6.27 (d, 0.2H, J = 2.9 \text{ Hz, } 1\alpha); ^{13}C \text{ NMR: (CDCl}_3, 400Hz), } \delta = 19.6 (\text{CH}_3, \text{OAc}), 19.6 (\text{CH}_3, \text{OAc}), 19.7 (\text{CH}_3, \text{OAc}), 19.8 (\text{CH}_3, \text{OAc}), 30.5 (\text{CH}_3, 6), 66.9 (\text{CH}, 5), 67.6 (\text{CH}, 2), 70.3 (\text{CH}, 4), 71.2 (\text{CH}, 3), 91.2 (\text{CH}, 1), 165.4 (\text{C=O, OAc}), 168.2 (\text{C=O, OAc}), 168.5 (\text{C=O, OAc}), 169.5 (\text{C=O, OAc}); \text{ LR-MS: ES}+ [M+Na]^+ = 355 (100%).

\[ \alpha-D-Galactopyranosyl bromide, 6-deoxy, 2,3,4-triacetate (273) \]

\[ \text{D-Galactopyranose, 6-deoxy, 1,2,3,4-tetracetate 272 (1.04 g, 3.10 mmol) was reacted using conditions detailed in the general procedure for bromination of acetylated monosaccharides, affording } \alpha-D-galactopyranosyl bromide, 6-deoxy, 2,3,4-triacetate 273 (0.67 g, yield = 60.8%) as a thick yellow oil. NMR spectroscopy showed that the product obtained was purely the } \alpha-\text{anomer.} \]

\[ ^1H \text{ NMR: (CDCl}_3, 400 \text{ Hz), } \delta = 1.15 (d, 3H, J = 6.5 \text{ Hz, } 6), 1.95 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.11 (s, 3H, OAc), 4.33 (q, 1H, J = 6.5 \text{ Hz, } 5), 4.96 (dd, 1H, J = 10.6, 3.9 \text{ Hz, } 2), 5.29 (dd, 1H, J = 3.3, 1.1 \text{ Hz, } 4), 5.34 (dd, 1H, J = 10.5, 3.4 \text{ Hz, } 3), 6.63 (d, 1H, J = 3.9 \text{ Hz, } 1); ^{13}C \text{ NMR: (CDCl}_3, 400 \text{ Hz), } \delta = 15.5 (\text{CH}_3, 6), 20.5 (\text{CH}_3, \text{OAc}), 20.5 (\text{CH}_3, \text{OAc}), 20.6 (\text{CH}_3, \text{OAc}), 67.8 (\text{CH}, 2), 68.4 (\text{CH}, 3), 69.8 (\text{CH}, 5), 70.0 (\text{CH}, 4), 89.3 \]
(CH, 1), 169.7 (C=O, OAc), 170.0 (C=O, OAc), 170.2 (C=O, OAc); **LR-MS**: ES+ [M+Na]^+ = 375 (100%), 377 (95%).

**D-Arabinono-Hex-5-enitol, 2,6-anhydro-1,5-dideoxy-, diacetate (274)**

\[
\begin{array}{c}
\text{AcO}_2 \\
\text{Br}^{\text{IV}} \\
\text{CuSO}_4, \text{Zn}
\end{array}
\rightarrow
\begin{array}{c}
\text{AcO}_2 \\
\text{AcO}
\end{array}
\]

\[\text{273} \quad \text{274}\]

\[\alpha-D-\text{Galactopyranosyl bromide, 6-deoxy, 2,3,4-triacetate 273 (76.2 mg, 0.22 mmol) was reacted using conditions detailed in the general glycal formation procedure. This reaction afforded D-arabinono-Hex-5-enitol, 2,6-anhydro-1,5-dideoxy-, diacetate 274 (16.6 mg, yield = 35.3%) as a thick yellow oil.}^{220}\]

**1H NMR**: (CDCl\textsubscript{3}, 400Hz), \(\delta = 1.20\) (d, 3H, \(J = 6.6\) Hz, 6), 1.95 (s, 3H, OAc), 2.10 (s, 3H, OAc), 4.15 (q, 1H, \(J = 6.6\) Hz, 5), 4.57 (dt, 1H, \(J = 6.3, 1.9\) Hz, 2), 5.21 (dt, 1H, \(J = 4.7, 1.2\) Hz, 4), 5.45-5.55 (m, 1H, 3), 6.40 (dd, 1H, \(J = 6.3, 1.8\) Hz, 1); **13C NMR**: (CDCl\textsubscript{3}, 400Hz), \(\delta = 15.5\) (CH\textsubscript{3}, 6), 19.7 (CH\textsubscript{3}, OAc), 19.9 (CH\textsubscript{3}, OAc), 64.1 (CH, 3), 65.3 (CH, 4), 70.6 (CH, 5), 97.3 (CH, 2), 145.1 (CH, 1), 169.4 (C=O, OAc), 169.7 (C=O, OAc); **LR-MS**: ES+ [M+Na]^+ = 237 (100%).

**D-Xylopyranose, 1,2,3,4-Tetraacetate (276)**

\[
\begin{array}{c}
\text{HO} \\
\text{HO} \\
\text{HO}
\end{array}
\rightarrow
\begin{array}{c}
\text{AcO}_2 \\
\text{AcO}_2 \\
\text{AcO}_2
\end{array}
\]

\[\text{275} \quad \text{276}\]

\[\text{D-Xylose 275 (1.50 g, 10 mmol) was reacted using conditions detailed in the general acetylation procedure. This reaction afforded D-xylopyranose, 1,2,3,4-tetraacetate 276 (3.54 g, yield = 99.2%) as a thick yellow oil. NMR spectroscopy showed that the product obtained was 4:1 }\beta:\alpha \text{ anomer.}^{221}\]
\textbf{\( ^1 \text{H NMR} \): (CDCl\textsubscript{3}, 300Hz), \( \delta = 1.94-2.18 \) (m, 12H, 4xOAc), 3.46 (dd, 0.8H, \( J = 12.0, 8.4 \) Hz, 5b\( \beta \)), 3.65 (t, 0.2H, \( J = 11.0 \) Hz, 5a\( \alpha \)), 3.87 (dd, 0.2H, \( J = 11.2, 5.9 \) Hz, 5a\( \alpha \)), 4.09 (dd, 0.8H, \( J = 12.0, 5.0 \) Hz, 5b\( \alpha \)), 4.87-5.01 (m, 2H, 2\( \alpha \), 2\( \beta \), 4\( \alpha \), 4\( \beta \)), 5.14 (t, 0.8H, \( J = 8.3 \) Hz, 3\( \beta \)), 5.40 (t, 0.2H, \( J = 9.8 \) Hz, 3a\( \alpha \)), 5.65 (d, 0.8H, \( J = 6.9 \) Hz, 1\( \beta \)), 6.20 (d, 0.2H, \( J = 3.7 \) Hz, 1\( \alpha \)); \textbf{\( ^{13} \text{C NMR} \): (CDCl\textsubscript{3}, 300Hz), \( \delta = 20.6 \) (CH\( _3 \), OAc), 20.6 (CH\( _3 \), OAc), 20.7 (CH\( _3 \), OAc), 20.8 (CH\( _3 \), OAc), 62.8 (CH\( _2 \), 5), 68.4 (CH, 4) 69.6 (CH, 2), 71.1 (CH, 3), 92.1 (CH, 1), 167.9 (C=O, OAc), 169.0 (C=O, OAc), 169.3 (C=O, OAc), 169.8, (C=O, OAc); \textbf{LR-MS}: ES+ [M+Na]\(^+\) = 341 (100%).

\textbf{\( \alpha-D \)-Xylopyranosyl bromide, 2,3,4-triacetate (276)}

\[\text{\small{\( \alpha-D \)-Xylopyranose, 1,2,3,4-tetraacetate 275 (1.62 g, 5.10 mmol) was reacted using conditions detailed in the general procedure for the bromination of acetylated monosaccharides, which afforded \( \alpha-D \)-xylopyranosyl bromide, 2,3,4-triacetate 276 (1.27 g, yield = 73.5\%) as a thick yellow oil. NMR spectroscopy showed that the product obtained was purely the \( \alpha \)-anomer.\( ^{221} \)}}\]

\textbf{\( ^1 \text{H NMR} \): (CDCl\textsubscript{3}, 300 Hz), \( \delta = 1.99 \) (s, 6H, 2xOAc), 2.04 (s, 3H, OAc), 3.81 (t, 1H, \( J = 11.2 \) Hz, 5a\( \alpha \)), 3.99 (dd, 1H, \( J = 11.3, 6.0 \) Hz, 5b\( \beta \)), 4.71 (dd, 1H, \( J = 9.8, 4.0 \) Hz, 2), 4.90-5.01 (ddd, 1H, \( J = 11.0, 9.6, 5.9 \) Hz, 4), 5.49 (t, 1H, \( J = 9.7 \) Hz, 3), 6.52 (d, 1H, \( J = 4.0 \) Hz, 1); \textbf{\( ^{13} \text{C NMR} \): (CDCl\textsubscript{3}, 300 Hz), \( \delta = 20.6 \) (3xCH\( _3 \), OAc), 62.5 (CH\( _2 \), 5), 68.1 (CH, 4) 69.5 (CH, 3), 70.8 (CH, 2), 87.7 (CH, 1), 169.8 (3xC=O, OAc).\)}}
\textbf{D-Xylosyl 3,4-diacetate (277)}

\begin{center}
\chemfig{\text{\(\alpha\text{-D-Xylopyranosyl bromide, 2,3,4-triacetate 276 (1.25 g, 3.69 mmol) was reacted using conditions detailed in the general glycal formation procedure. This reaction afforded D-xylosyl 3,4-diacetate 277 (0.44 g, yield = 60.1\%) as a thick yellow oil.}^{222}}}
\end{center}

\textbf{Rf:} 0.8 (1:1 ethyl acetate:hexane); \textbf{\(^1\text{H NMR:}\) (CDCl\textsubscript{3}, 300Hz), \(\delta = 2.00\) (s, 3H, OAc), 2.03 (s, 3H, OAc), 3.91 (dd, 1H, \(J = 12.1\) Hz, 5a), 4.08 (dd, 1H, \(J = 0.9\) Hz, 5b), 4.80-4.95 (m, 3H, 2, 3, 4), 6.54 (d, 1H, \(J = 5.5\) Hz, 1); \textbf{\(^{13}\text{C NMR:}\) (CDCl\textsubscript{3}, 300Hz), \(\delta = 20.9\) (CH\(_3\), OAc), 21.1 (CH\(_3\), OAc), 63.4 (CH, 3), 63.6 (CH\(_2\), 5), 67.2 (CH, 4), 97.4 (CH, 2), 148.8 (CH, 1), 169.8 (C=O, OAc), 169.9, (C=O, OAc); \textbf{LR-MS ES+ [MNa]+} = 223.0 (100\%).}

\textbf{1,1'-Bi-2-naphthol (137)}

\begin{center}
\chemfig{\text{\(\text{Naphth-2-ol 136 (0.20 g, 1.39 mmol) was dissolved in toluene (30 ml), before FeCl\textsubscript{3}.6H\textsubscript{2}O (0.75 g, 2.77 mmol) was added. The mixture was heated then heated to 50 °C and stirred for 4 hours. The resulting mixture was filtered and the solvent was removed in vacuo, affording 1,1-bi-2-naphthol 137 (0.16 g, yield = 81.6\%) as off white crystals.}^{223}}}
\end{center}

\textbf{Rf = 0.55 (4:1 hexane:ethyl acetate); \textbf{\(^1\text{H NMR}\)(CDCl\textsubscript{3}, 400Hz), \(\delta = 7.18\) (d, 2H, \(J = 8.6\) Hz), 7.20-7.45 (m, 6H), 7.93 (d, 2H, \(J = 8.0\) Hz), 8.02 (d, 2H, \(J = 8.6\) Hz).}
Resolution of \((R)\) and \((S)\) bi-naphth-2-ol \((137)\)

Resolution of enantiomers performed as described by Schanz\(^{165}\). Racemic 1,1-binaphth-2-ol \(137\) (5.0 g, 17.30 mmol) and \((R,R)\)-diaminocyclohexane (DACH) \(207\) (2.0 g, 17.50 mmol) was dissolved in toluene (75 mL). The solution was then heated to reflux under an inert N\(_2\) atmosphere for 2 hours. The solution was allowed to cool to room temperature and the \([((R)\text{-BINOL.}(R,R)\text{DACH}.\text{toluene})\] complex proceeded to crystallise out of solution. Hexane was added to aid crystallisation, before the precipitate was collected \(\text{via}\) filtration and washed with toluene (2 x 20 mL). The precipitate was then dissolved in aqueous HCl (26 ml, 0.1M), before being extracted with ethyl acetate (3 x 25 mL). The solution was then evaporated to dryness \(\text{in vacuo}\), affording \(R\) 1,1'-binaphth-2-ol \(137\) (1.54 g, yield = 31.1\%) as off white crystals. The filtrate was also collected and evaporated to dryness \(\text{in vacuo}\), affording \(S\) 1,1'-bi-naphthol \(137\) (1.42 g, yield = 28.4\%) as off white crystals. Both \(R\) and \(S\) binaphth-2-ol enantiomers were confirmed \(\text{via}\) optical rotation in THF.

\(R\)-2,2-Binapthol, \([\alpha]_D^{20} = 33.8^\circ\) \((c = 1, \text{CHCl}_3)\), Lit = 35.5\(^\circ\)\(^{224}\)

\(S\)-2,2-Binapthol, \([\alpha]_D^{20} = -36.1^\circ\) \((c = 1, \text{CHCl}_3)\), Lit = -35.5\(^\circ\)\(^{224}\)

\(^1\)H NMR for both \(R\) and \(S\) enantiomers were identical to racemic 1,1-binaphth-2-ol \(137\).
(R)-2,2′-bis(methoxymethoxy)-1,1′-binaphthalene (138)

Reactions was performed as described by Wu et al.\textsuperscript{166} NaH, (0.30 g, 73 mmol) was washed with hexane and then mixed with dry THF (15 ml) under an inert N\textsubscript{2} atmosphere. The solution was then cooled to 0 °C, before (R)-binapthol 137 (0.76 g, 2.62 mmol) in dry THF (8 ml) was added dropwise. The solution was then stirred for 1 hr at 0 °C before being allowed to warm to room temperature. Solution was then re-cooled to 0 °C before chloromethylether (0.50 g, 6.21 mmol) was added dropwise. Solution was allowed to warm to room temperature and left for 18 hrs. Reaction was quenched using a saturated NH\textsubscript{4}Cl solution (20 ml), before the solution was extracted with DCM (3 x 20 ml). The organic extracts were combined, washed with water (50 mL) and brine (50 mL) before being dried with MgSO\textsubscript{4}. Solvent was evaporated to dryness \textit{in vacuo} to afford a crude brown oil, which was purified \textit{via} column chromatography (7:1 hexane/ethyl acetate) to afford 2,2′-bismethoxymethyl-(R)-napthyl 138 (0.64 g, yield = 64.5%) as off white crystals.\textsuperscript{166}

\[
\text{R}_{f} = 0.7 \text{ (1:1 hexane:ethyl acetate); } ^1H \text{ NMR: } (\text{CDCl}_3, 300 \text{ Hz}), \delta = 3.08 \text{ (s, 6H), 4.90 (d, 2H, } J = 6.8 \text{ Hz), 5.00 (d, 2H, } J = 6.8 \text{ Hz), 7.05-7.32 (6H, m), 7.50 (d, 2H, } J = 9.0 \text{ Hz), 7.80 (d, 2H, } J = 8.3 \text{ Hz), 7.90 (d, 2H, } J = 9.0 \text{ Hz); } ^{13}C \text{ NMR: } (\text{CDCl}_3, 300 \text{ Hz}), \delta = 55.8 \text{ (CH}_3), 95.1 \text{ (CH}_2), 117.2 \text{ (CH), 123.1 (C), 124.1 (CH), 125.5 (CH), 126.4 (CH), 128.2 (CH), 129.4 (CH), 130.13 (C), 134.2 (C), 152.7 (C); } \text{LR-MS: } \text{ES+ [MNa}^+] = 398.5; \text{ FTIR (thin film): } 3590, 2950, 1610, 1590, 1500, 1480, 1220, 1120, 1010, 810, 750.\]
(R)-3,3'-dibromo-2,2'-bis(methoxymethoxy)-1,1'-binaphthalene (139)

Reactions was performed as described by Wu et al. Bismethoxymethyl-(R)-napthalene 138 (0.78 g, 2.14 mmol) was dissolved in Et₂O (25 ml), before the dropwise addition of nBuLi (2.5 ml, 1M solution in hexane) under an inert N₂ atmosphere. The solution was stirred for 3 hrs, before THF (17 ml) was added. The solution was then stirred for a further 1 hr, before the addition of dibromo-tetrachlororoethane (1.30 g, 4.04 mmol) at 0 °C. The solution was then stirred for 15 mins, before the addition of saturated NH₄Cl solution (25 ml). The organic layer was extracted with Et₂O (2 x 25 ml), washed with brine (50 mL) and dried using MgSO₄. The solvent was evaporated to dryness to afford a black oil, which was purified using column chromatography (5:1 hexane:ethyl acetate).

3,3-dibromo bismethoxymethyl-(R)-napthalene 139 (0.50 g, yield = 59.0%) was afforded as off white crystals.

Rᶠ = 0.7 (1:1 hexane:ethyl acetate); ¹H NMR (CDCl₃, 400Hz), δ = 2.61 (s, 6H), 4.91 (m, 4H), 7.25-7.35 (m, 4H), 7.46 (t, 2H, J = 7.4 Hz), 7.84 (d, 2H, J = 8.2 Hz), 8.33 (s, 2H); ¹³C NMR (CDCl₃, 400 Hz), δ = 56.5 (CH₃), 99.5 (CH₂), 117.8 (C), 126.5 (CH), 127.0 (CH), 127.5 (2xCH), 131.9 (C), 133.4 (CH), 133.5 (C), 150.5 (C); LR-MS ES⁺ [MNa]⁺ = 556 (100%), 555 (50%).
(R)-3,3'-dibromo-[1,1'-binaphthalene]-2,2'-diol (140)

Reactions was performed as described by Wu et al. 166 3,3-dibromo bismethoxymethyl-(R)-napthyl 139 (0.50 g, 0.95 mmol) and Amberlyst 15 resin (2.0 g) was added to a 1:1 THF:MeOH (15 ml) solvent mixture. The mixture was then heated to reflux under an inert N₂ atmosphere for 2 hrs. The mixture was allowed to cool to room temperature before the Amberlyst 15 resin was removed via filtration. The solution was evaporated to dryness in vacuo, affording a brown oil. This oil was then purified using column chromatography (5:1 hexane:ethyl acetate) affording (R)-3,3'-dibromo-[1,1'-binaphthalene]-2,2'-diol 140 (0.38 g, yield = 90.1%) as off white crystals. 166

**Rf** = 0.7 (1:1 hexane:ethyl acetate); **¹H NMR** (CDCl₃, 400 Hz): δ = 7.13 (d, 2H, J = 8.4 Hz), 7.34 (t, 2H, J = 8.0 Hz), 7.41 (t, 2H, J = 7.1 Hz), 7.84 (d, 2H, J = 8.1 Hz), 8.28 (d, 2H, J = 6.7 Hz); **¹³C NMR** (CDCl₃, 400 Hz): δ = 112.3 (C), 114.7 (C), 124.7 (CH), 124.9 (CH), 127.4 (CH), 127.6 (CH), 129.8 (CH), 132.8 (C), 148.1 (CH); **LR-MS** ES+ [M⁺]= 453.3 (40%), [M+H⁺]= 454.4 (100%); **FTIR** (thin film): 3510, 1760, 1540, 1450, 1230, 1200, 1170, 980, 760, 740.

(R)-2-phenyl-2H-pyran-4(3H)-one (144)

Reaction performed as described by Du et al. 164 3,3-dibromo binaphthol 140 (28.4 mg, 64.0 µmol) was dissolved in dry toluene (5 ml), before the addition of Et₂Zn (0.1 mL, 1M
solution in hexane) under an N₂ atmosphere. Solution was stirred for 30 mins before being cooled to -75 °C. Danishefskis diene 142 (96.8 mg, 0.56 mmol) and benzaldehyde 143 (71.2 mg, 0.67 mmol) were then added and solution was allowed to warm to room temperature, before being stirred for 24 hrs. The reaction was quenched using saturated NH₄Cl solution (10 ml) and extracted with EtOAc (2 x 10 ml). The solution was evaporated to dryness in vacuo affording a crude yellow oil, which was purified using column chromatography (5:1 hexane:ethyl acetate). Pure 5-phenyl-pyran-3-one 144 (46.2 mg, yield = 48.6%) was afforded, with the appearance of a yellow oil. Analysis via chiral HPLC showed an 11:2 mixture of (S) to (R) enantiomers, ee = 71.0%. (R)-5-phenyl-pyran-3-one = 11.9 mins, (S)-5-phenyl-pyran-3-one = 13.9 mins (5µ 4.6 x 250 Chiralpak OD column, 9:1 hexane:isopropanol).¹⁶⁴

\[
\text{R}_f = 0.4 (4:1 \text{ hexane:ethyl acetate}); \quad ^1\text{H NMR (CDCl}_3, 300 \text{ Hz}), \delta = 2.82 (\text{bd, 1H, } J = 14.4 \text{ Hz}, 4a), 2.87 (\text{bd, 1H, } J = 14.4 \text{ Hz}, 4b), 5.36 (\text{dd, 1H, } J = 14.4, 3.5 \text{ Hz}, 5), 5.46 (\text{dd, 1H, } J = 6.0, 1.2 \text{ Hz}, 2), 7.32 (\text{m, 5H, } 7, 8, 9) 7.42 (\text{d, 1H, } J = 6.0 \text{ Hz}, 1); \quad ^{13}\text{C NMR (CDCl}_3, 300 \text{ Hz}), \delta = 43.4 (\text{CH}_2, 4), 81.1 (\text{CH}, 5), 107.4 (\text{CH}, 2), 126.1 (\text{CH}, 7), 128.8 (\text{CH}, 9), 129.0 (\text{CH}, 8), 137.8 (\text{C}, 6), 163.3 (\text{CH}, 1), 192.3 (\text{C}, 3); \quad \text{LR-MS ES}^+ [\text{M+Na}^+] = 197.2 (100%).
\]

(2R,3S,4R,6R)-2-(acetoxymethyl)-6-(neopentyloxy)tetrahydro-2H-pyran-3,4-diyl diacetate (146) and (2R,3S,4R,6S)-2-(acetoxymethyl)-6-(neopentyloxy)tetrahydro-2H-pyran-3,4-diyl diacetate (147)

![Reaction scheme](image)

Reaction was performed as described by Silva et al.¹⁶⁷ Glucal triacetate 135 (68.0 mg, 0.25 mmol), Ph₃P.HBr (68.4 mg, 0.12 mmol) and activated 4Å molecular sieves were mixed with toluene (4 mL) under an inert N₂ atmosphere. 2,2-dimethyl propanol (22.0 mg, 0.25 mmol) was then added, before the reaction was stirred for 24 hrs. The reaction
mixture was then filtered and then evaporated to dryness in vacuo, affording a yellow oil. The resulting precipitate was purified by column chromatography (4:1 hexane:ethyl acetate) affording a 4:1 mix of 1-(R)-dimethyl propanoxy-(3R,4S,5R)-5-(acetoxy methyl) tetrahydro-2H-pyran-3,4-diy diacetate 146 and 1-(S)-dimethyl propanoxy-(3R,4S,5R)-5-(acetoxy methyl) tetrahydro-2H-pyran-3,4-diy diacetate 147 as a colourless oil (Combined 44.3 mg, yield = 49.1%). NMR data was obtained for the major α-isomer 147 only.

\[\text{H NMR (CDCl}_3, 300 \text{ Hz): } \delta = 0.87 \text{ (s, 9H, 9), 1.95 (s, 3H, OAc), 1.98 (s, 3H, OAc), 2.03 (s, 3H, OAc), 1.78 (dd, 1H, } J = 12.5, 3.4 \text{ Hz, 2a), 2.19 (dd, 1H, } J = 12.5, 5.6 \text{ Hz, 2b), 2.93 (d, 1H, } J = 9.0 \text{ Hz, 7a), 3.25 (d, 1H, } J = 9.0 \text{ Hz, 7b), 3.88 (ddd, 1H, } J = 10.1, 4.8, 2.2 \text{ Hz, 5), 4.00 (dd, 1H, } J = 12.2, 2.1 \text{ Hz, 6a), 4.22 (dd, 1H, } J = 12.2, 2.1 \text{ Hz, 6b), 4.84 (d, 1H, } J = 3.4 \text{ Hz, 1), 4.92 (t, 1H, } J = 10.0 \text{ Hz, 4), 5.22-5.31 (m, 1H, 3); }^{13}\text{C NMR (CDCl}_3, 300 \text{ Hz): } \delta = 20.8 \text{ (2xCH}_3, 2xOAc), 21.0 \text{ (CH}_3, \text{ OAc), 26.7 (3xCH}_3, 9), 31.7 (C, 8), 35.1 \text{ (CH}_2, 2), 62.5 \text{ (CH}_2, 6), 67.8 \text{ (CH, 3), 69.3 (CH, 5), 69.6 (CH, 4), 78.2 (CH}_2, 7), 97.3 \text{ (CH, 1), 170.0 (2xC=O, OAc), 170.8 (C=O, OAc); LR-MS ES+ [M+Na]^+ = 383, (100%); HR-MS: ES+ calc for C}_{17}H_{28}O_{8}Na 383.1682, found 383.1799.}

\((2R,3S,6S)-3\text{-acetoxy-6-(neopentyloxy)-3,6-dihydro-2H-pyran-2-yl)methyl acetate (148) and } (2R,3S,6R)-3\text{-acetoxy-6-(neopentyloxy)-3,6-dihydro-2H-pyran-2-yl)methyl acetate (149)}

Reaction was performed as described by Gorityala et al.\textsuperscript{68} Glucal triacetate 135 (122.6 mg, 0.45 mmol), phosphoric acid (50 µL, 0.09 mmol) and 2,2-dimethyl propanol 145 (40.0 mg, 0.45 mmol) were dissolved in DCM (1.5 mL). The solution was stirred for 40 mins, before the solution was diluted with DCM, washed with saturated NaHCO\textsubscript{3}, dried with MgSO\textsubscript{4} and evaporated to dryness in vacuo, affording a crude yellow oil. The oil
was purified using flash column chromatography (3:1 hexane:ethyl acetate), affording a 4:1 ratio of ((2R,3S,6S)-3-acetoxy-6-(neopentyloxy)-3,6-dihydro-2H-pyran-2-yl) methylacetate 148 and ((2R,3S,6R)-3-acetoxy-6-(neopentyloxy)-3,6-dihydro-2H-pyran-2-yl) methylacetate 149 (combined, 79 mg, yield = 58.4%), with the appearance of colourless oil. NMR data was obtained for the major α-isomer.225

\[
\text{Rf: 0.64 (1:1 ethyl acetate:hexane); } ^1\text{H NMR: (CDCl}_3 300Hz), \delta = 0.85 (s, 9H, 9), 2.02 (s, 3H, OAc), 2.03 (s, 3H, OAc), 3.07 (d, 1H, } J = 8.8 \text{ Hz, 6a), 3.38-3.44 (m, 1H, 6b), 3.86-4.07 (m, 1H, 5), 4.09-4.20 (m, 1H, 7), 4.92 (s, 1H, 1), 5.24 (d, 1H, } J = 9.6 \text{ Hz, 4), 5.72-5.84 (m, 2H, 2, 3); } ^13\text{C NMR: (CDCl}_3 300Hz), \delta = 20.8 (\text{CH}_3, \text{OAc}), 21.1 (\text{CH}_3, \text{OAc}), 26.7 (3\times\text{CH}_3, 9), 31.8 (\text{C}, 8), 63.1 (\text{CH}_2, 7), 65.5 (\text{CH}, 4), 67.0 (\text{CH}, 5), 79.0 (\text{CH}_2, 6), 94.6 (\text{CH}, 1), 128.1 (\text{CH}, 2), 128.8 (\text{CH}, 3), 170.3 (\text{C}=\text{O}, \text{OAc}), 170.8 (\text{C}=\text{O}, \text{OAc}); \]
\[\text{LR-MS: } [\text{M+Na}]^+ = 355.\]

**Fusidic Acid (166)**

Fusidic acid sodium salt 278 (50 mg, 92.8 µmol) was dissolved in a 1:1 water:DCM (1 mL) mixture, before concentrated hydrochloric acid (2-3 drops) was added to the solution. The organics were extracted with DCM (3 x 1 mL), dried over MgSO₄ and evaporated to dryness in vacuo, affording fusidic acid 166 (47.4 mg, yield = 98.8%) as a white powder.59
**1H NMR**: (400 Hz, CDCl₃), δ = 0.83-0.87 (m, 6H, 18, 28), 0.90 (s, 3H, 19), 0.95-1.15 (m, 2H, 7a, 6a), 1.21 (d, 1H, J = 14.2 Hz, 15a), 1.31 (s, 3H, 29), 1.45 (bd, 1H, J = 12.5 Hz, 1a), 1.47 (d, 1H, J = 1.0 Hz, 9), 1.5-1.55 (m, 5H, 4, 6b, 26), 1.60 (s, 3H, 27), 1.63-1.72 (m, 2H, 7b, 2a), 1.73-1.85 (m, 2H, 12a, 2b), 1.90 (s, 3H, OAc), 1.95-2.15 (m, 4H, 1b, 5, 23, 15b), 2.25 (dt, 1H, J = 13.1, 2.9 Hz, 12b), 2.31-2.45 (m, 2H, 22), 2.99 (bd, 1H, J = 11.1 Hz, 13), 3.69 (bd, 1H, J = 2.4 Hz, 3), 4.28 (bd, 1H, J = 1.3 Hz, 11), 5.03 (bt, 1H, J = 7.2 Hz, 24), 5.81 (d, 1H, J = 8.3 Hz, 16); **13C NMR**: (400 Hz, CDCl₃), δ = 15.9 (CH₃, 28), 17.8 (CH₃, 18), 17.9 (CH₃, 26), 20.6 (CH₃, OAc), 20.8 (CH₂, 6), 22.8 (CH₃, 19), 24.1 (CH₃, 29), 25.7 (CH₃, 27), 28.4 (CH₂, 23), 28.8 (CH₂, 22), 29.9 (CH₂, 2), 30.2 (CH₂, 1), 32.3 (CH₂, 7), 35.5 (CH₂, 12), 36.1 (CH, 5), 36.2 (CH, 4), 37.0 (C, 10), 38.9 (CH₂, 15), 39.4 (C, 8), 44.3 (CH, 13), 48.7 (C, 14), 49.2 (CH, 9), 68.3 (CH, 11), 71.5 (CH, 3), 74.4 (CH, 16), 123.0 (CH, 24), 129.5 (C, 20), 132.7 (C, 25), 151.0 (C, 17), 170.6 (C=O, OAc), 174.0 (C, 21); **LR-MS**: ES⁺ [M+Na⁺]: 539 (100%), ES⁻ [M-H⁻]: 515 (100%); **IR** (powder) νmax: 3484, 2931, 2869, 1712, 1549, 1371, 1270, 1024, 962, 603, 530 cm⁻¹; [α]D²⁰ = -8.6° (c =1, CHCl₃), Lit = -9°.²²⁶

**Neopentyl cyclohexylcarboxylate (217)**

![Chemical structure](image)

2,2-Dimethylpropanol 145 (25 mg, 0.28 mmol), cyclohexanoic acid 216 (50 mg, 0.56 mmol) and sulphonyl chloride resin 215 (0.23 g, 1.75 mmol/g) were mixed with DCM (2
Methyl imidazole (94.2 mg, 1.12 mmol) was then added before the reaction vessel was sealed and stirred for 24 hrs at room temperature. The mixture was diluted with DCM (2.5 mL) and shaken with Amberlyst-15 resin for 10 mins. The resin was then filtered off, before the mixture was shaken with polystyrene divinylbenzene amine resin for a further 10 mins. The mixture was then filtered again, before being evaporated to dryness in vacuo, affording neopentyl cyclohexanecarboxylate 217 (50.6 mg, yield = 91.2%) as a colourless oil.²²⁷

¹H NMR: (400Hz, CDCl₃), δ = 0.87 (s, 9H, 7), 1.1-1.3 (m, 3H, 3a, 4a), 1.38 (dq, 2H, J = 11.9, 3.0 Hz, 2a), 1.50-1.62 (m, 1H, 4b), 1.64-1.75 (m, 2H, 3b), 1.8-1.9 (m, 2H, 2b), 2.26 (tt, 1H, J = 11.2, 3.0 Hz, 1), 3.69 (s, 2H, 6); ¹³C NMR: (400Hz, CDCl₃), δ = 25.3 (2xCH₂, 3), 25.7 (CH₂, 4), 26.5 (3xCH₃, 8), 28.8 (2xCH₂, 2), 31.4 (C, 7), 42.9 (CH, 1), 73.4 (CH₂, 6), 182.3 (C, 5); LR-MS ES⁺ [M+Na]⁺ = 221 (100%).

Fusidic acid-21-methyl ester; (Z)-methyl 2-((3R, 4S, 5S, 8S, 9S, 10S, 11R, 13R, 14S, 16S)-16-acetoxy-3,11-dihydroxy-4, 8, 10, 14-tetramethyldecahydro-1H-cyclopenta[a]phenanthren-17(2H,10H,14H)-ylidene)-6-methylhept-5-enoate (221)

Fusidic acid 168 (50.0 mg, 96.8 µmol) was dissolved in 2:1 toluene:methanol (0.6 mL) mixture. Trimethylsilyl diazomethane (0.06 mL, 0.12 mmol) was added to the solution at 0 °C and under a N₂ atmosphere. The mixture was allowed warm to room temperature and stirred for 8 hrs. The reaction was quenched using 10% acetic acid solution, extracted
with ethyl acetate (3 x 1.5 mL), dried with MgSO₄ and then evaporated to dryness in vacuo affording 221 (49.7 mg, yield = 96.7%) as a white crystalline solid.⁶⁹

**¹H NMR**: (400 Hz, CDCl₃), δ = 0.83 (s, 3H, 18), 0.85 (d, 3H, J = 6.9 Hz, 28), 0.90 (s, 3H, 19), 0.94 -1.12 (m, 2H, 7a, 6a), 1.20-1.24 (m, 1H, 15a), 1.30 (s, 3H, 29), 1.44 (bd, 1H, J = 12.0 Hz, 1a), 1.49 (d, 1H, J = 1.8 Hz, 9), 1.5-1.57 (m, 5H, 4, 6b, 26), 1.60 (s, 3H, 27), 1.63-1.72 (m, 2H, 7b, 2a), 1.73-1.83 (m, 2H, 12a, 2b), 1.91 (s, 3H, OAc), 1.93-2.15 (m, 5H, 1b, 5, 15b, 23), 2.25 (dt, 1H, J = 13.1, 3.2 Hz, 12b), 2.29-2.47 (m, 2H, 22), 2.96 (bd, 1H, J = 10.8 Hz, 13), 3.57 (s, 3H, CO₂Me), 3.68 (bd, 1H, J = 2.4 Hz, 3), 4.27 (bd, 1H, J = 1.8 Hz, 11), 5.02 (tt, 1H, J = 7.2, 1.2 Hz, 24), 5.77 (d, 1H, J = 8.4 Hz, 16); **¹³C NMR**: (400 Hz, CDCl₃), δ = 16.0 (CH₃, 28), 17.8 (2 x CH₃, 18, 26), 20.8 (CH₃, Ac), 20.8 (CH₂, 6), 21.0 (CH₃, 19), 24.1 (CH₃, 29), 25.8 (CH₃, 27), 28.3 (CH₂, 23), 28.9 (CH₂, 22), 29.9 (CH₂, 2), 30.2 (CH₂, 1), 32.3 (CH₂, 7), 35.5 (CH₂, 12), 36.1 (CH, 5), 36.2 (CH, 4), 37.0 (C, 10), 39.0 (CH₂, 15), 39.4 (C, 8), 43.9 (CH, 13), 48.7 (C, 14), 49.2 (CH, 9), 51.4 (CO₂Me), 68.3 (CH, 11), 71.4 (CH, 3), 74.4 (CH, 16), 123.1 (CH, 24), 130.4 (C, 20), 132.6 (C, 25), 148.2 (C, 17), 170.4 (C, 21), 170.8 (C=O, Ac); **LR-MS**: ES+ [M+Na]+: 553 (100%); **HR-MS**: ES+ calc for C₃₂H₅₀O₆Na 533.3500, found 533.3499; **IR** (powder) ν_max: 3473, 3326, 2923, 1734, 1639, 1431, 1689, 1372, 1236, 1012, 818 cm⁻¹; [α]D²⁰ = -13.2° (c = 0.5, CHCl₃), Lit = -14°.⁷²⁶
General method for C-3 esterification of fusidic acid

Fusidic acid-21-methyl ester 218, the carboxylic acid (1.4 eq) and sulphonyl chloride resin 215 (1.4 eq) were mixed with DCM (0.5 mL). Methyl imidazole was then added before the reaction vessel was sealed and heated to 50 °C for 24 hrs. The mixture was allowed to cool to room temperature, diluted with DCM (2.5 mL) and shaken with Amberlyst-15 resin for 10 mins. The resin was then filtered off, before the mixture was shaken with polystyrene divinyl benzene amine resin for 10 mins. The mixture was then filtered again before being evaporated to dryness in vacuo.

Fusidic acid-21-methyl ester, 3-cyclohexanecarboxylate; 29-Nordammara-17(20),24-dien-21-oic acid, 16-(cyclohexanecarboxylate)-3-(hexanoate)-11-hydroxy-, (acetyloxy)methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β,17Z)- (9CI) (219)

Fusidic acid-21-methyl ester 218 (10.1 mg, 19.0 µmol) was reacted with cyclohexanoic acid 216 (3.4 mg, 26.6 µmol) using conditions detailed in general esterification method, which afforded 219 (10.9 mg, yield = 91.1%) as a white powder.
**1H NMR:** (400 Hz, CDCl₃), δ = 0.75 (d, 3H, J = 6.7 Hz, 28), 0.78-0.82 (m, 1H, 6a), 0.84 (s, 3H, 18), 0.91 (s, 3H, 19), 0.94 -1.12 (m, 1H, 7a), 1.20-1.27 (m, 2H, 15a, 33a), 1.31 (s, 3H, 29), 1.35-1.45 (m, 2H, 1a, 32a), 1.49 (d, 1H, J = 1.6 Hz, 9), 1.53 (s, 3H, 26), 1.55-1.59 (m, 3H, 6b, 34a, 34b), 1.60 (s, 3H, 27), 1.63-1.75 (m, 4H, 2a, 5, 7b, 33b), 1.80-1.90 (m, 4H, 2b, 12a, 23, 32b), 1.92 (s, 3H, OAc), 1.93-2.19 (m, 3H, 1b, 4, 12b), 2.20-2.32 (m, 1H, 31), 2.32-2.48 (m, 2H, 22), 2.96 (bd, 1H, J = 11.4 Hz, 13), 3.58 (s, 3H, CO₂Me), 4.27 (bd, 1H, J = 2.0 Hz, 11), 4.85 (bd, 1H, J = 2.6 Hz, 3), 5.02 (tt, 1H, J = 7.2, 1.2 Hz, 24), 5.77 (d, 1H, J = 8.2 Hz, 16); **13C NMR:** (400 Hz, CDCl₃), δ = 14.7 (CH₃, 28), 16.8 (CH₃, 26), 17.0 (CH₃, 18), 19.5 (CH₂, 6), 20.0 (CH₃, OAc), 21.5 (CH₃, 19), 23.3 (CH₃, 29), 24.3 (CH₂, 33), 24.7 (CH₂, 34), 24.8 (CH₃, 27), 27.3 (CH₂, 2), 27.8 (CH₂, 22), 28.2 (CH₂, 32), 28.7 (CH₂, 23), 30.0 (CH₂, 1), 31.9 (CH₂, 7), 33.8 (CH, 5), 34.7 (CH₂, 12), 36.0 (C, 10), 37.0 (CH, 4), 38.1 (CH₂, 15), 38.4 (C, 10), 41.6 (CH, 31), 42.9 (CH, 13), 47.7 (C, 14), 48.0 (CH, 9), 50.4 (CO₂Me), 67.3 (CH, 11), 72.6 (CH, 3), 73.4 (CH, 16), 122.0 (CH, 24), 129.5 (C, 20), 131.6 (C, 25), 147.1 (C, 17), 169.7 (C=O, 21), 174.6 (C=O, OAc), 179.6 (C=O, 30); **LR-MS:** ES+ [M+Na]+: 663 (100%), ES- [M+Cl]⁻: 675 (26%); **HR-MS:** ES+ calc for C₉₉H₆₆O₂Na 663.4232, found 663.4228; **IR** (powder) νmax: 3534, 2933, 1707, 1448, 1376, 1251, 1193, 1020, 904, 731 cm⁻¹; [α]D²⁰ = -23.0° (c = 0.2, MeOH).
Fusidic acid-21-methyl ester, 3-hexanoate; 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetylxy)-3-(hexanoate)-11-hydroxy-, (acetylxy)methyl ester, (3a,4a,8a,9β,11α,13α,14β,16β,17Z)- (9CI) (220)

Fusidic acid-21-methyl ester 218 (10.5 mg, 19.8 μmoles) was reacted with hexanoic acid (3.2 mg, 27.7 μmoles), using conditions detailed in general esterification method, which afforded 220 (11.2 mg, yield = 90.2%) as a white powder.

$^1$H NMR: (400 Hz, CDCl$_3$), $\delta$ = 0.75 (d, 3H, $J$ = 6.7 Hz, 28), 0.79-0.82 (m, 3H, 35), 0.83 (s, 3H, 18), 0.91 (s, 3H, 19), 0.94 -1.12 (m, 2H, 6a, 7a), 1.20-1.28 (m, 5H, 15a, 32, 33), 1.31 (s, 3H, 29), 1.45-1.59 (m, 6H, 1a, 6b, 9, 34), 1.60 (s, 3H, 27), 1.62-1.77 (m, 2H, 2a, 2b, 7b), 1.80 (dd, 2H, $J$ = 12.9, 2.7 Hz, 12a) 1.91 (s, 3H, OAc), 1.97-2.15 (m, 4H, 1b, 4, 23a, 23b), 2.20-2.29 (m, 3H, 5, 12b, 15b), 2.30-2.47 (m, 1H, 22), 2.96 (d, 1H, $J$ = 10.8 Hz, 13), 3.58 (s, 3H, CO$_2$Me), 4.26 (bd, 1H, $J$ = 1.5 Hz, 11), 4.87 (bd, 1H, $J$ = 2.5 Hz, 3), 5.02 (tt, 1H, $J$ = 7.2, 1.3 Hz, 24), 5.79 (d, 1H, $J$ = 8.4 Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta$ = 13.9 (CH$_2$, 35), 15.7 (CH$_3$, 28), 17.8 (CH$_3$, 26), 18.0 (CH$_3$, 18), 20.5 (CH$_2$, 6), 21.0 (CH$_3$, OAc), 22.5 (CH$_3$, 19), 24.4 (CH$_3$, 29), 24.4 (CH$_2$, 34), 24.8 (CH$_3$, 27), 27.4 (CH$_2$, 2), 28.3 (CH$_2$, 23), 28.9 (CH$_2$, 22), 29.7 (CH$_2$, 1), 31.1 (CH$_2$, 32), 31.4 (CH$_2$, 33), 32.9 (CH$_2$, 7), 34.7 (CH, 5), 34.8 (CH$_2$, 31), 35.7 (CH$_2$, 12), 37.0 (C, 10), 37.9 (CH, 4),
39.0 (C, 8), 39.1 (CH₂, 15), 43.9 (CH, 13), 47.7 (C, 14), 49.0 (CH, 9), 51.5 (CH₃, CO₂Me), 68.3 (CH, 11), 73.9 (CH, 3), 74.4 (CH, 16), 123.0 (CH, 24), 130.5 (C, 20), 132.6 (C, 25), 148.1 (C, 17), 170.4 (C=O, 21), 170.7 (C=O, OAc), 173.6 (C=O, 30); LRMS: ES⁺ [M+Na]⁺: 651(75%); IR (powder) ν_max: 3394, 2957, 2923, 2856, 1722, 1438, 1376, 1256, 1015, 789 cm⁻¹; [α]_D²⁰ = -21.4° (c = 0.2, MeOH).

**Fusidic acid-21-methyl ester, 3-(3,3-dimethyl-butanoate); 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetyloxy)-3-(3,3-dimethyl-butanoate)-11-hydroxy-, (acetyloxy)methyl ester, (3a,4α,8α,9β,11α,13α,14β,16β,17Z)-(9CI) (221)**

Fusidic acid-21-methyl ester 218 (9.9 mg, 18.7 µmol) was reacted with 3,3-dimethyl butanoic acid (3.0 mg, 26.2 µmol) using conditions detailed in the general esterification method, which afforded 221 (10.9 mg, 92.3% yield) as a white solid.

**¹H NMR:** (400 Hz, CDCl₃), δ = 0.77 (d, 3H, J = 6.7 Hz, 28), 0.84 (s, 3H, 18), 0.92 (s, 3H, 19), 0.95-1.24 (m, 4H, 2a, 6b, 7a, 15a), 1.29 (s, 3H, 29), 1.32-1.44 (m, 2H, 1a, 9), 1.49 (s, 9H, 33), 1.53 (s, 3H, 26), 1.54-1.58 (m, 1H, 6a), 1.60 (s, 3H, 27), 1.63-1.77 (m, 3H, 2b, 5, 7b), 1.80 (dd, 1H, J = 12.8, 2.4 Hz, 12a), 1.91 (s, 3H, OAc), 1.93-2.10 (m, 5H, 3H, 26).
1b, 4, 15b, 23a, 23b), 2.16 (s, 2H, 31), 2.18-2.29 (m, 1H, 12b), 2.30-2.47 (m, 2H, 22), 2.96 (d, 1H, J = 11.7 Hz, 13), 3.58 (s, 3H, CO₂Me), 4.27 (bd, 1H, J = 1.0 Hz, 11), 4.87 (bd, 1H, J = 2.6 Hz, 3), 5.01 (tt, 1H, J = 7.1, 1.3 Hz, 24), 5.79 (d, 1H, J = 8.3 Hz, 16); ¹³C NMR: (400 Hz, CDCl₃), δ = 14.8 (CH₃, 28), 16.7 (CH₃, 26), 16.9 (CH₃, 18), 19.6 (CH₂, 6), 20.0 (CH₃, OAc), 21.7 (CH₃, 19), 23.1 (CH₃, 29), 24.7 (CH₃, 27), 26.4 (CH₂, 2), 27.2 (CH₂, 23), 28.3 (CH₂, 22), 28.7 (3xCH₃, 33), 29.7 (C, 32), 30.0 (CH₂, 1), 31.7 (CH₂, 7), 33.7 (CH, 5), 34.7 (CH₂, 12), 35.9 (C, 10), 36.8 (CH, 4), 38.1 (CH₂, 15), 38.4 (C, 8), 42.8 (CH, 13), 47.7 (CH₂, 31), 47.8 (C, 14), 48.1 (CH, 9), 50.4 (CH₃, CO₂Me), 67.2 (CH, 11), 73.0 (CH, 3), 73.4 (CH, 16), 122.0 (CH, 24), 129.5 (C, 20), 131.6 (C, 25), 147.1 (C, 17), 169.4 (C=O, 21), 169.7 (C=O, OAc), 171.2 (C=O, 30); LR-MS: ES⁺ [M+Na]⁺: 651 (69%); HR-MS: ES⁺ calc for C₃₈H₆₀O₇Na 651.4232, found 651.4256; IR (powder) νmax: 3452, 2956, 2927, 1720, 1255, 1012, 797 cm⁻¹; [α]D²⁰ = -16.5° (c = 0.2, MeOH).

Fusidic acid-21-methyl ester, 3-isobutyrate; 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acytyloxy)-3-[isobutyrate]-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β, 17Z)- (9CI) (224)

Fusidic acid-1-methyl ester 218 (16.0 mg, 30.1 µmol) was reacted with isobutyric acid (3.8 µL, 45.6 µmol) using conditions detailed in the general esterification method, which afforded 224 (11.5 mg, 74.1% yield) as a white solid.
$^1$H NMR: (400 Hz, CDCl$_3$), $\delta = 0.76$ (d, 3H, $J = 6.7$ Hz, 28), 0.84 (s, 3H, 18), 0.92 (s, 3H, 19), 0.95-1.10 (m, 3H, 2a, 6a, 7a), 1.12 (d, 6H, $J = 6.8$ Hz, 31), 1.19 (s, 3H, 29), 1.20-1.29 (m, 1H, 15a), 1.29 (s, 3H, 29), 1.34-1.52 (m, 3H, 1a, 6b, 9), 1.53 (s, 3H, 26), 1.60 (s, 3H, 27), 1.62-1.77 (m, 3H, 2b, 5, 7b), 1.80 (dd, 1H, $J = 12.8$, 2.5 Hz, 12a), 1.91 (s, 3H, OAc), 1.94-2.16 (m, 5H, 1b, 4, 15b, 23a, 23b), 2.23 (dt, 1H, $J = 13.3$, 2.9 Hz, 12b), 2.30-2.49 (m, 2H, 22), 2.46-2.58 (m, 1H, 32), 2.96 (d, 1H, $J = 10.7$ Hz, 13), 3.58 (s, 3H, CO$_2$Me), 4.26 (bd, 1H, $J = 2.1$ Hz, 11), 4.84 (bd, 1H, $J = 2.7$ Hz, 3), 5.02 (dt, 1H, $J = 7.2$, 2.6 Hz, 24), 5.79 (d, 1H, $J = 8.4$ Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta = 14.6$ (CH$_3$, 28), 16.7 (CH$_3$, 26), 16.9 (CH$_3$, 18), 18.2 (2xCH$_3$, 33), 19.5 (CH$_2$, 6), 20.0 (CH$_3$, OAc), 21.5 (CH$_3$, 19), 23.3 (CH$_3$, 29), 24.7 (CH$_3$, 27), 26.3 (CH$_2$, 2), 27.2 (CH$_2$, 23), 27.9 (CH$_2$, 22), 28.6 (CH$_2$, 31), 30.0 (CH$_2$, 1), 31.9 (CH$_2$, 7), 33.6 (CH, 5), 34.7 (CH$_2$, 12), 36.0 (C, 10), 36.9 (CH, 4), 38.0 (CH$_2$, 15), 38.4 (C, 8), 39.8 (CH, 32), 42.9 (CH, 13), 47.7 (C, 14), 48.0 (CH, 9), 50.4 (CH$_3$, CO$_2$Me), 67.3 (CH, 11), 72.7 (CH, 3), 73.4 (CH, 16), 122.0 (CH, 24), 129.5 (C, 20), 131.6 (C, 25), 147.1 (C, 17), 169.4 (C=O, 21), 169.7 (C=O, OAc), 171.2 (C=O, 30); LRMS: ES$^+$ [M+Na]$^+$: 623 (100%) HR-MS: ES$^+$ calc for C$_{38}$H$_{60}$O$_7$Na 623.4532, found 623.3924; $[\alpha]_b^{20} = 8.2^\circ$ (c = 0.2, MeOH).
Fusidic acid-21-methyl ester, 3-benzoate; (29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetyloxy)-3-[benzoyloxy]-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β, 17Z) - (9CI) (225)

Fusidic acid-1-methyl ester 218 (9.7 mg, 18.2 μmol) was reacted with benzoic acid (3.1 mg, 25.5 μmol) using conditions detailed in general esterification method, which afforded 225 (9.2 mg, yield = 81.7%) as a white powder.

$^1$H NMR: (400 Hz, CDCl$_3$), $\delta$ = 0.83 (d, 3H, J = 6.9 Hz, 28), 0.87 (s, 3H, 28), 0.97 (s, 3H, 19), 1.00-1.25 (m, 4H, 2a, 6a, 7a, 15a), 1.39 (s, 3H, 29), 1.40-1.58 (m, 3H, 1a, 6b, 9), 1.49 (d, 1H, J = 1.5 Hz, 9), 1.60 (s, 3H, 27), 1.70-1.90 (m, 4H, 2b, 5, 7b, 12a), 1.97 (s, 3H, OAc), 2.00-2.30 (m, 5H, 1b, 4, 15b, 23a, 23b), 2.30-2.49 (m, 1H, 12b), 2.99 (d, 1H, J = 11.1 Hz, 13), 3.58 (s, 3H, CO$_2$Me), 4.28 (d, 1H, J = 1.8 Hz, 11), 5.01 (tt, 1H, J = 7.2, 1.3 Hz, 24), 5.13 (bd, 1H, J = 2.5 Hz, 3), 5.79 (d, 1H, J = 8.4 Hz, 16), 7.38 (t, 1H, J = 7.6 Hz, 34), 7.49 (t, 2H, J = 7.4 Hz, 33), 7.99 (bd, 2H, J = 7.2 Hz, 32); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta$ = 14.8 (CH$_3$, 28), 16.7 (CH$_3$, 26), 17.0 (CH$_3$, 18), 19.6 (CH$_2$, 6), 20.0 (CH$_3$, OAc), 21.5 (CH$_3$, 19), 23.5 (CH$_3$, 29), 24.7 (CH$_3$, 27), 26.4 (CH$_2$, 2), 27.2 (CH$_2$, 23), 27.9 (CH$_2$, 22), 30.1 (CH$_2$, 1), 31.9 (CH$_2$, 7), 34.0 (CH, 5), 34.7 (CH$_2$, 12), 36.0 (C, 10), 37.1 (C, 4), 38.0 (CH$_2$, 15), 38.5 (C, 8), 42.9 (CH, 13), 47.7 (C, 14), 48.0 (CH, 9), 50.4 (CH$_3$, 28), 58.0 (CH$_2$, 27), 67.9 (OAc), 125.0 (C, 1), 131.8 (C, 6), 134.0 (C, 17), 135.0 (C, 24), 136.0 (C, 11), 137.0 (C, 25), 140.0 (C, 8), 145.0 (C, 28), 161.8 (OAc), 167.0 (C, 19), 196.0 (C, 18).
CO₂Me), 67.2 (CH, 11), 73.3 (CH, 3), 73.9 (CH, 16), 122.0 (CH, 24), 127.4 (2xCH, 33), 128.4 (2xCH, 32), 129.4 (C, 31), 129.9 (C, 20), 131.6 (C, 34), 131.8 (C, 25), 147.1 (C, 17), 165.1 (C=O, 30), 169.4 (C=O, OAc), 169.7 (C=O, 21); **LR-MS:** ES⁺ [M+Na]⁺: 657 (100%); IR (powder) νmax: 3543, 2957, 2919, 1726, 1693, 1443, 1375, 1256, 1092, 1015, 799, 766, 529 cm⁻¹; [α]D²⁰ = -30.0° (c = 0.4, MeOH).

Fusidic acid-21-methyl ester, 3-cinnamate; 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetyloxy)-3-[cinamate]-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β, 17Z)- (9CI) (229)

Fusidic acid-1-methyl ester 218 (10.2 mg, 19.2 µmol) was reacted with *trans*-cinnamic acid (4.0 mg, 26.9 µmol) using conditions detailed in the general esterification method, which afforded 229 (6.6 mg, yield = 52.2%) as an off-white solid.

**¹H NMR:** (400 Hz, CDCl₃), δ = 0.85 (d, 3H, J = 6.8 Hz, 28), 0.86 (s, 3H, 18), 0.95 (s, 3H, 19), 0.94-1.18 (m, 2H, 6a, 7a), 1.20-1.24 (m, 1H, 15a), 1.38 (s, 3H, 29), 1.47-1.52 (m, 2H, 1a, 9), 1.53 (s, 3H, 26), 1.54-1.58 (m, 1H, 6b), 1.60 (s, 3H, 27), 1.65-1.88 (m,
6H, 2a, 2b, 5, 7b, 12a, 23a), 1.92 (s, 3H, OAc), 1.93-2.20 (m, 4H, 1b, 4, 15b, 23b), 2.25 (dt, 1H, J = 13.0, 3.0 Hz, 12b), 2.30-2.50 (m, 2H, 22), 2.99 (d, 1H, J = 10.5 Hz, 13), 3.58 (s, 3H, CO₂Me), 4.28 (bd, 1H, J = 1.9 Hz, 11), 5.02 (m, 2H, 3, 24), 5.80 (d, 1H, J = 8.4 Hz, 16), 6.39 (d, 1H, J = 16.0 Hz, 31), 7.33 (m, 3H, 34, 36) 7.45 (m, 2H, 35), 7.62 (d, 1H, J = 16.0 Hz, 32); ¹³C NMR: (400 Hz, CDCl₃), δ = 14.7 (CH₃, 28), 16.7 (CH₃, 26), 17.0 (CH₃, 18), 19.6 (CH₂, 6), 20.0 (CH₃, OAc), 21.6 (CH₃, 19), 23.5 (CH₃, 29), 24.7 (CH₃, 27), 26.4 (CH₂, 2), 27.2 (CH₂, 23), 27.9 (CH₂, 22), 30.1 (CH₂, 1), 31.8 (CH₂, 7), 34.0 (CH, 5), 34.8 (CH₂, 12), 36.0 (C, 10), 36.9 (CH, 4), 38.1 (CH₂, 15), 38.5 (C, 8), 42.9 (CH, 13), 47.7 (C, 14), 48.1 (CH, 9), 51.4 (CH₃, CO₂Me), 67.2 (CH, 11), 73.2 (CH, 3), 73.4 (CH, 16), 117.8 (CH, 31), 122.0 (CH, 24), 127.1 (2xCH, 35), 127.3 (CH, 36), 127.9 (2xCH, 34), 129.5 (C, 33), 130.6 (C, 20), 132.5 (C, 25), 143.4 (CH, 32), 147.1 (C, 17), 165.1 (C, 30), 169.4 (C, OAc), 169.7 (C, 21); LRMS: ES⁺ [M+Na]⁺: 683 (36%); IR (powder) νmax: 3423, 2957, 2923, 2851, 1712, 1457, 1376, 1256, 1020, 794 cm⁻¹; [α]D²⁰ = 44.2° (c = 0.1, MeOH).

Fusidic acid-21-methyl ester, 3-([p]-chlorobenzoate); 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetyloxy)-3-[(4-chlorobenzoyl)oxy]-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β,17Z)-(9CI) (233)

Fusidic acid-1-methyl ester 218 (10.0 mg, 18.9 µmol) was reacted with 4-chloro benzoic acid (2.8 mg, 28.3 µmol), using conditions detailed in general esterification method, which afforded 233 (7.6 mg, yield = 60.3%) as a white solid.
$^1$H NMR: (400 Hz, CDCl$_3$), $\delta = 0.82$ (d, 3H, $J = 6.6$ Hz, 28), 0.86 (s, 3H, 18), 0.96 (s, 3H, 19), 1.00-1.35 (m, 3H, 6a, 7a, 15a), 1.37 (s, 3H, 29), 1.37 (s, 3H, 29), 1.43-1.58 (m, 3H, 1a, 6b, 9), 1.60 (s, 3H, 27), 1.65-1.90 (m, 5H, 5, 7b, 12a, 23), 1.91 (s, 3H, OAc), 1.95-2.28 (m, 6H, 1b, 2a, 2b, 4, 12b, 15b), 2.35-2.50 (m, 1H, 22), 2.98 (bd, 1H, $J = 11.3$ Hz, 13), 3.58 (s, 3H, OAc), 4.27 (bd, 1H, $J = 1.7$ Hz), 5.01 (tt, 1H, $J = 7.2, 1.4$ Hz, 24), 5.12 (bd, 1H, $J = 2.4$ Hz, 3), 5.80 (d, 1H, $J = 8.4$ Hz, 16), 7.36 (d, 2H, $J = 8.6$ Hz, 31), 7.91 (d, 2H, $J = 8.6$ Hz, 32); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta = 15.9$ (CH$_3$, 28), 17.8 (CH$_3$, 26), 18.0 (CH$_3$, 18), 20.6 (CH$_2$, 6), 21.0 (CH$_3$, OAc), 22.4 (CH$_3$, 19), 24.5 (CH$_3$, 29), 25.7 (CH$_3$, 27), 27.4 (CH$_2$, 23), 28.1 (CH$_2$, 2), 28.9 (CH$_2$, 22), 31.1 (CH$_2$, 1), 32.8 (CH$_2$, 7), 35.1 (CH, 5), 35.9 (CH$_2$, 12), 37.0 (C, 10), 38.1 (CH, 4), 38.8 (CH$_2$, 15), 39.5 (C, 8), 43.9 (CH, 13), 48.8 (C, 14), 49.0 (CH, 9), 51.5 (CH$_3$, CO$_2$Me), 68.2 (CH, 11), 74.3 (CH, 16), 75.3 (CH, 3), 122.9 (CH, 24), 128.8 (CH, 32), 129.3 (C, 20), 130.8 (CH, 33), 132.6 (C, 25), 132.7 (C, 31), 139.2 (C, 34), 148.0 (C, 17), 166.7 (C, 30), 169.6 (C, OAc), 173.2 (C, 21); LR-MS: ES$^+$ [M+Na]$^+$: 691(100%) (Cl$^{15}$), 693 (55%) (Cl$^{37}$); IR (powder) $\nu_{max}$: 3438, 2923, 1717, 1376, 1260, 1092, 1015, 760, 525 cm$^{-1}$; $[\alpha]_D^{20} = 30.1^\circ$ (c = 0.1, MeOH).
Fusidic acid-21-methyl ester, 3-(p-bromobenzoate); 29-Nordammara-17(20),24-dien-21-oic acid, 16-(acetyloxy)-3-[(4-bromobenzoyl)oxy]-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β,17α) (9CI) (235)

Fusidic acid-1-methyl ester 218 (12.1 mg, 22.8 µmol) was reacted with 4-bromo benzoic acid (6.4 mg, 31.9 µmol) using conditions detailed in the general esterification method, which afforded 235 (13.4 mg, 82.1% yield) as a pale brown solid.²²⁸

¹H NMR: (300 Hz, CDCl₃), δ = 0.82 (d, 3H, J = 6.7 Hz, 28), 0.86 (s, 3H, 18), 0.96 (s, 3H, 19), 1.00-1.51 (m, 5H, 1a, 6a, 7a, 9, 15a), 1.52 (s, 3H, 26), 1.60 (s, 3H, 27), 1.62-1.90 (m, 6H, 2a, 2b, 5, 6b, 7b, 12a), 1.92 (s, 3H, OAc), 1.95-2.30 (m, 5H, 1b, 4, 12b, 15b, 23), 2.30-2.50 (m, 2H, 22), 2.98 (d, 1H, J = 10.7 Hz, 13), 3.58 (s, 3H, CO₂Me), 4.28 (d, 1H, J = 1.7 Hz, 11), 5.01 (tt, 1H, J = 7.1, 1.3 Hz, 24), 5.12 (bd, 1H, J = 2.4 Hz, 3), 5.81 (d, 1H, J = 8.3 Hz, 16), 7.56 (d, 2H, J = 8.6 Hz, 33), 7.83 (d, 2H, J = 8.6 Hz, 32); ¹³C NMR: (300 Hz, CDCl₃), δ = 15.8 (CH₃, 28), 17.8 (CH₃, 26), 18.0 (CH₃, 18), 20.6 (CH₂, 6), 21.0 (CH₃, OAc), 22.6 (CH₃, 19), 24.4 (CH₃, 29), 25.7 (CH₃, 27), 27.4 (CH₂, 2), 28.3 (CH₂, 23), 28.9 (CH₂, 22), 31.2 (CH₂, 1), 32.8 (CH₂, 7), 35.1 (CH, 5), 35.9 (CH₂, 12), 37.0 (C,
10), 38.2 (CH, 4), 39.1 (CH₂, 15), 39.5 (C, 8), 43.9 (CH, 13), 48.8 (C, 14), 49.1 (CH, 9), 51.4 (CH₃, CO₂Me), 68.2 (CH, 11), 74.3 (CH, 16), 75.4 (CH, 3), 123.0 (CH, 24), 127.9 (C, 34), 129.9 (C, 20), 130.6 (C, 31), 131.0 (2xCH, 33), 131.8 (2xCH, 32), 132.6 (C, 25), 148.0 (C, 17), 165.4 (C, 30), 170.4 (C, OAc), 170.7 (C, 21); LRMS: ES⁺ [M+Cl]⁺: 749 (100%) (Br⁸¹Cl³⁵) & (Br⁷⁹Cl³⁷), 747 (35%) (Br⁷⁹Cl³⁵), 751 (27%) (Br⁸¹Cl³⁷); HRMS: ES⁺ [M⁺]: calc for C₃₉H₅₃O₇BrCl 747.2668, found 747.2667; IR (powder) νmax: 3548, 2923, 2856, 1722, 1688, 1433, 1375, 1289, 1106, 1010, 880, 760 cm⁻¹; [α]D²⁰ = 29.8° (c = 0.1, MeOH).

**General method for para-cinnamic acid synthesis**

![General method for para-cinnamic acid synthesis](image)

The aldehyde (0.25 g) was dissolved in a 1:1 toluene and pyridine (4 mL) mixture, before malonic acid (2 eq) was added. The solution was then heated to 95 °C and stirred for 2 hrs, before being quenched with 5M HCl (10 mL) which causes precipitation. The solid was filtered and washed with cold hexane (3 x 10 mL).

**p-Bromo trans-cinnamic acid (280)**

*p*-Bromo benzadehyde (0.25 g, 1.35 mmol) was reacted with malonic acid 279 (0.28 g, 2.70 mmol) using conditions detailed in the general *para*-cinnamic acid formation method, which afforded 280 (0.25 g, 82.3% yield) with the appearance of a brown solid.²²⁹

¹H NMR: (300 Hz, CDCl₃), δ = 6.57 (d, 1H, J = 16.0, 6.7 Hz, 3), 7.57 (d, 1H, J = 16.1, 6.7 Hz, 2), 7.61-7.69 (m, 4H, 5, 6); ¹³C NMR: (300 Hz, CDCl₃), δ = 120.1 (CH, 3), 130.1 (2 x CH, 5), 131.8 (2xCH, 6), 133.5 (C, 4), 142.6 (CH, 2), 167.4 (C, 7), 206.4 (C=O, 1);
LR-MS: ES⁺ [M-H]⁺: 225 (100%) (Br79), 227 (94%) (Br81); IR (powder) νmax: 2827, 1679, 1626, 1486, 1424, 1308, 1284, 1274, 1072, 813, 707, 544, 487 cm⁻¹.

*p-Nitro trans*-cinnamic acid (281)

*p-Nitro benzadehyde* (0.25 g, 1.65 mmoles) was reacted with malonic acid 279 (0.34 g, 3.30 mmoles), using conditions detailed in the general *para*-cinnamic acid formation method, which afforded 281 (0.29 g, 91.9% yield) as an off white solid.230

![Image of *p-Nitro trans*-cinnamic acid (281)]

1H NMR: (300Hz, DMSO), δ = 6.75 (d, 1H, J = 16.1 Hz, 2), 7.70 (d, 1H, J = 16.1 Hz, 3), 7.98 (d, 2H, J = 8.7 Hz, 6), 8.24 (d, 2H, J = 8.8 Hz, 5); 13C NMR: (300Hz, DMSO), δ = 122.9 (CH, 2), 123.9 (CH, 6), 129.3 (CH, 5), 141.7 (C, 4), 141.3 (CH, 3), 147.0 (C, 7), 173.4 (C, 1); LR-MS: ES⁺ [M-H]⁺: 192 (22%), 385 (100%); IR (powder) νmax: 3029, 2673, 1688, 1596, 1515, 1429, 1337, 1207, 900, 847, 760, 707, 554 cm⁻¹.

(3R, 4S, 5S, 8S, 9S, 10S, 11R, 13R, 14S, 16S, Z)-3,11-dihydroxy-17-(6-iodo-7,7-dimethyl-2-oxooxepan-3-ylidene)-4,8,10,14-tetramethylhexadecahydro-1H-cyclopenta[a]phenanthren-16-yl acetate (240)

![Image of (3R, 4S, 5S, 8S, 9S, 10S, 11R, 13R, 14S, 16S, Z)-3,11-dihydroxy-17-(6-iodo-7,7-dimethyl-2-oxooxepan-3-ylidene)-4,8,10,14-tetramethylhexadecahydro-1H-cyclopenta[a]phenanthren-16-yl acetate (240)]

Fusidic acid 166 (20 mg, 37.7 µmol), NaHCO₃ (6.3 mg, 75.5 µmol) and water (1 mL) was added to a reaction vessel and stirred until all solids had dissolved. Chloroform (1 mL) was then added to the solution and it was cooled to 0 °C. Iodine was added (19.2 mg, 75.5 µmol) and the reaction was stirred at 0 °C for 6 hrs. The mixture was then
extracted with chloroform (5 mL), washed with saturated Na₂CO₃ solution until
colourless and then washed again with water (5 mL) and brine (5 mL) solutions. The
organic layer was dried over MgSO₄ and evaporated to dryness in vacuo affording crude
yellow oil. This crude product was then purified by reverse phase HPLC. HPLC run on a
Lichrosorb RP-18 10 μm 25x0.78 mm column, flow of 3 ml/min, solvent system of 8:2
MeOH:H₂O. This resulted in 4 product peaks with retention times of 12.6 min for product
2 (2.6 mg, yield = 10.7%), 23.4 min, 28.1 min for product 3 (1.1 mg, yield = 4.5%) and
33.8 min for 240 (13.6 mg, yield = 56.1%), all with the appearance of a white solid. ¹³C
NMR was attempted for all products but insufficient amounts of product were isolated
for products 2 and 3.

¹H NMR: (400 Hz, CDCl₃), δ = 0.86 (d, 3H, J = 6.8 Hz, 28), 0.89 (s, 3H, 18), 0.91 (s,
3H, 19), 0.95-1.15 (m, 2H, 6a, 7a), 1.27 (d, 2H, J = 14.2 Hz, 15a) 1.31 (s, 3H, 29), 1.44
(ddd, 1H, J = 11.9, 3.9, 2.5 Hz, 1a), 1.48-1.60 (m, 4H, 4, 6b, 9, 12a), 1.70 (s, 3H, 26),
1.71 (s, 3H, 27), 1.75-1.93 (m, 2H, 2b, 7b), 1.95 (s, 3H, OAc), 1.99-2.15 (m, 3H, 1b, 5,
15b) 2.20-2.40 (m, 2H, 12b, 23a), 2.52-2.66 (m, 1H, 23b), 2.82 (ddd, 1H, J = 14.6, 5.7,
2.0 Hz, 22b), 3.03 (d, 1H, J = 11.7 Hz, 13), 3.70 (bd, 1H, J = 2.5 Hz, 3), 4.2-4.4 (m, 2H,
11, 24), 5.80 (d, 1H, J = 8.5 Hz, 16); ¹³C NMR: (400 Hz, CDCl₃), δ = 14.9 (CH₃, 28),
17.2 (CH₃, 18), 19.7 (CH₂, 6), 19.8 (CH₃, OAc), 20.5 (CH₃, 26), 21.6 (CH₃, 19), 23.1
(CH₃, 29), 28.7 (CH₂, 2), 28.9 (CH₂, 22), 29.3 (CH₂, 1), 31.6 (CH₂, 7), 34.4 (CH₂, 12),
34.6 (CH₃, 27), 35.1 (CH, 5), 35.3 (CH, 4), 35.9 (CH, 24), 36.1 (C, 10), 37.5 (CH₂, 15),
38.5 (C, 8), 39.2 (CH₂, 23), 42.4 (CH, 13), 48.2 (C, 14), 48.9 (CH, 9), 67.3 (CH, 11), 70.3
(CH, 3), 72.9 (CH, 16), 83.4 (C, 25), 128.0 (C, 20), 148.9 (C, 17), 168.9 (C, 21), 169.1
(C=O, OAc); LR-MS: ES⁺ [M+Na]⁺, 665 (100%); HR-MS: ES⁺ [M-H]⁺: calc for
C₃₁H₄₇O₈NaI 665.2310 found 665.2304; IR (powder) ν_max: 3393, 2925, 1706, 1371,
1258, 1074, 917, 797, 729 cm⁻¹; [α]D²⁰ = -14.7° (c = 0.2, MeOH).
Product 2

$^1\text{H NMR}$: (400 Hz, CDCl$_3$), $\delta$ = 0.84-0.88 (m, 6H), 0.90 (s, 3H), 0.92-1.30 (m, 6H), 1.32 (s, 3H), 1.4-1.5 (m, 2H) 1.51 (s, 3H), 1.52-1.60 (m, 4H), 1.69 (s, 3H), 1.7-1.9 (m, 3H), 1.95 (s, 3H), 1.98 (s, 2H), 2.01-2.20 (m, 4H), 2.20-2.51 (m, 2H), 2.92 (bd, 1H, $J$ = 13.9 Hz), 3.04 (bd, 1H, $J$ = 13.0 Hz), 3.70 (bd, 1H, $J$ = 2.0 Hz), 4.27 (bd, 1H, $J$ = 2.3 Hz), 4.56 (t, 1H, $J$ = 3.5 Hz), 5.74 (dd, 1H, $J$ = 5.7, 8.6 Hz); LR-MS: ES$^+$ [M+Na]$^+$, 665 (100%); HR-MS: ES$^+$ [M-H]$^+$: calc for C$_{31}$H$_{47}$O$_6$NaI 665.2310 found 665.2304.

Product 3

$^1\text{H NMR}$: (400 Hz, CDCl$_3$), $\delta$ = 0.86 (d, 3H, $J$ = 6.7 Hz), 0.92 (s, 3H), 0.95 (s, 3H), 0.95-1.15 (m, 6H), 1.19 (s, 3H), 1.31 (s, 3H), 1.41-1.61 (m, 6H), 1.65-1.85 (m, 3H), 1.90 (s, 3H), 1.91 (s, 3H), 1.93 (m, 6H), 2.20-2.51 (m, 2H), 2.96 (bd, 1H, $J$ = 14.6 Hz), 3.08 (bd, 1H, $J$ = 12.3 Hz), 3.56 (dd, $J$ = 2.9, 10.8 Hz), 3.71 (bd, 1H, $J$ = 2.9 Hz), 4.29 (bd, 1H, $J$ = 1.8 Hz), 5.86 (d, 1H, $J$ = 8.1 Hz); LR-MS: ES$^+$ [M+Na]$^+$, 665 (100%); HR-MS: ES$^+$ [M-H]$^+$: calc for C$_{31}$H$_{47}$O$_6$NaI 665.2310 found 665.2304.

Fusidic acid 3,11-diacetate; 29-/ordammara-17(20),24-dien-21-oic acid, 3,11,16-tris(acetyloxy)-, methyl ester, (3α,4α,8α,9b,11α,13α,14β,16β,17Z)- (9CI) (247)

Fusidic acid 166 (14.6 mg, 28.3 µmol) was dissolved in pre-dried pyridine (0.4 mL), before acetic anhydride (0.1 mL) was added. The solution was mixed at room temperature for 48 hrs, before a few drops of 2M HCl (0.3 mL) was added. The reaction was then extracted with EtOAc (3 x 1.5 mL) and dried with MgSO$_4$, before being
evaporated to dryness in vacuo. This reaction afforded triacetate 247 (12.4 mg, yield = 73.3%) as a brown solid.\textsuperscript{231}

$^1$H NMR: (400 Hz, CDCl$_3$), $\delta$ = 0.75 (d, 3H, $J = 6.7$ Hz, 28), 0.87 (s, 3H, 18), 0.91 (s, 3H, 19), 0.94-1.17 (m, 2H, 6a, 7a), 1.18 (s, 1H, 1a), 1.20-1.28 (m, 1H, 15a), 1.29 (s, 3H, 29), 1.50 (s, 3H, 26), 1.52-1.58 (m, 1H, 1b ), 1.59 (s, 3H, 27), 1.61-1.81 (m, 7H, 1b, 2a, 2b, 4, 7b, 9, 12a), 1.88 (s, 3H, OAc), 1.90-1.96 (m, 1H, 12b), 1.97 (s, 3H, OAc), 2.00 (s, 3H, OAc), 2.01-2.10 (m, 2H, 5, 23b), 2.13-2.40 (m, 2H, 12b, 15b), 2.80 (bd, 1H, $J = 10.6$ Hz, 13), 4.85 (bd, 1H, $J = 2.3$ Hz, 3), 5.00 (tt, 1H, $J = 1.2$, 7.3 Hz, 24), 5.22 (bd, 1H, $J = 2.0$ Hz, 11), 5.82 (d, 1H, $J = 8.4$ Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta$ = 14.5 (CH$_3$, 28), 16.6 (CH$_3$, 18), 16.9 (CH$_3$, 26), 19.4 (CH$_2$, 6), 19.6 (CH$_3$, OAc), 20.3 (CH$_3$, OAc), 20.8 (CH$_3$, OAc), 21.4 (CH$_3$, 19), 23.0 (CH$_3$, 29), 24.7 (CH$_3$, 27), 26.2 (CH$_2$, 2), 27.1 (CH$_2$, 23), 27.7 (CH$_2$, 22), 28.7 (CH$_2$, 1), 31.0 (CH$_2$, 7), 31.7 (CH$_2$, 12), 32.6 (CH, 4), 35.8 (C, 10), 36.7 (CH, 5), 37.8 (CH$_2$, 15), 38.6 (C, 8), 43.5 (CH, 13), 46.8 (CH, 9), 47.5 (C, 14), 69.6 (CH, 11), 73.1 (CH, 3), 73.2 (CH, 16), 121.6 (CH, 24), 127.4 (C, 20), 131.8 (C, 25), 147.0 (C, 17), 169.2 (C=O, OAc), 169.6 (C=O, OAc), 169.8 (C=O, OAc), 174.9 (C=O, 21); LRMS: ES$^+$ [M-H]: 599 (100%), [M+Cl]$^+$: 635 (12%); HR-MS: ES$^+$ [M-H]: calc for C$_{35}$H$_{51}$O$_8$ 599.3589 found 599.3580; [a]$_D^{20} = -27.2^\circ$ (c = 0.5, CDCl$_3$). Lit = -21$^\circ$.\textsuperscript{231}
Fusidic acid-21-methyl ester, 3-acetate; 29-Nordammara-17(20),24-dien-21-oic acid, 3,16-bis(acetyloxy)-11-hydroxy-, methyl ester, (3α,4α,8α,9β,11α,13α,14β,16β,17Z)-(9CI) (272)

Fusidic acid-21-methyl ester 218 (10.5 mg, 19.8 μmol) was dissolved in pyridine (0.4 mL), before acetic anhydride (0.1 mL) was added. The solution was mixed at room temperature for 12 hrs, before 2M HCl (0.3 mL) was added. The reaction was then extracted with EtOAc (3 x 1.5 mL) and dried with MgSO₄. The solution was reduced to dryness in vacuo, which afforded diacetate 282 (10.4 mg, 91.7% yield) as a brown solid.²³²

¹H NMR: (400 Hz, CDCl₃), δ = 0.79 (d, 6H, J = 6.7 Hz, 26, 27), 0.83-0.87 (m, 6H, 18, 28), 0.91 (s, 3H, 19), 0.95-1.15 (m, 2H, 7a, 6a), 1.21 (dd, 1H, J = 14.2, 6.7 Hz, 15a, 23), 1.31 (s, 3H, 29), 1.35-1.6 (m, 1a, 9, 4, 6b, 25), 1.63-1.72 (m, 2H, 7b, 2a), 1.73-1.85 (m, 2H, 12a, 2b), 1.91 (s, 3H, OAc), 1.95-2.15 (m, 4H, 1b, 5, 24), 2.22 (dt, 1H, J = 13.1, 2.5 15b), 2.31-2.45 (m, 2H, 12b, 22), 2.98 (d, 1H, J = 11.2 Hz, 13), 3.70 (bd, 1H, J = 2.5 Hz, 3), 4.28 (bd, 1H, J = 1.7 Hz, 11), 5.80 (d, 1H, J = 8.4 Hz, 16); ¹³C NMR: (300 Hz, CDCl₃), δ = 14.5 (CH₃, 28), 16.7 (CH₃, 26), 16.9 (CH₃, 18), 19.5 (CH₂, 6), 20.0 (CH₃,
OAc), 20.3 (CH₃, OAc), 21.5 (CH₃, 19), 23.3 (CH₃, 29), 24.7 (CH₃, 24.5), 26.3 (CH₂, 2), 27.2 (CH₂, 23), 27.9 (CH₂, 22), 28.7 (CH₂, 1), 31.7 (CH₂, 7), 33.7 (CH, 4), 34.7 (CH₂, 12), 35.9 (C, 10), 36.7 (CH, 5), 38.1 (CH₂, 15), 38.4 (C, 8), 42.8 (CH, 13), 47.7 (C, 14), 48.0 (CH, 9), 50.4 (CH₃, CO₂Me), 67.2 (CH, 11), 73.1 (CH, 3), 73.4 (CH, 16), 122.0 (CH, 24), 129.5 (C, 20), 131.6 (C, 25), 147.1 (C, 17), 169.4 (C, OAc), 169.7 (C, OAc), 169.9 (C, 21); **LRMS**: ES⁺ [M-H]⁺: 599 (100%), [M+Cl]⁺: 635 (12%), [M+Na]⁺: 595 (100%); **HR-MS**: ES⁺ [M+Na]⁺: calcd for C₃₄H₅₂O₇Na 595.3611 Found 595.3641; [α]D²⁰ = -23.3° (c = 0.5, CHCl₃), Lit = -26°.²²⁶

**18-Nor-5α,8α,9β,13α,14β-androstane-3,11,17-trione, 16β-acetate-4α,8,14-trimethyl-(8Cl) (249)**

Fusidic acid (20.0 mg, 37.7 µmol) and methanol (1 mL) was treated with O₃, at -40 °C and stirred for 85 minutes. N₂ was then bubbled through the solution for 10 minutes, before dimethylsulfide (40 mL) was added and the solution was stirred for a further 24 hrs. The product was then extracted with diethyl ether (3 x 40 mL) and washed with water (40 mL), which afforded 181 (13.4 mg, 88.9% yield) as white solid.

**¹H NMR**: (400 Hz, CDCl₃), δ = 0.98-1.02 (m, 6H, 28, 18), 1.10 (s, 3H, 19), 1.12-1.18 (m, 1H, 7a), 1.20 (s, 3H, 29), 1.47-1.49 (m, 2H, 1a, 15a), 1.56 (dd, 1H, J = 13.1, 6.2 Hz, 6a), 1.75 (td, 1H, J = 12.2, 1.4 Hz, 5), 1.83-2.08 (m, 2H, 12a, 6b), 2.09 (s, 3H, OAc),
2.10-2.23 (m, 1H, 4), 2.28-2.48 (m, 3H, 1b, 2a, 9), 2.49-2.57 (m, 1H, 15b), 2.64-2.73 (m, 1H, 12b), 2.80 (dd, 1H, J = 12.3, 5.4 Hz, 13), 5.34 (d, 1H, J = 10.1 Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta$ = 14.1 (CH$_3$, 28), 17.0 (CH$_3$, 19), 20.9 (CH$_3$, OAc), 21.0 (CH$_2$, 6), 22.4 (CH$_3$, 18), 24.4 (CH$_3$, 29), 29.7 (CH$_2$, 7), 32.3 (CH$_2$, 12), 34.9 (CH$_2$, 15), 36.7 (CH$_2$, 1), 36.8 (C, 10), 37.9 (CH$_2$, 2), 41.4 (C, 8), 44.7 (CH, 4), 45.6 (C, 14), 46.5 (CH, 5), 49.1 (CH, 13), 59.1 (CH, 9), 71.8 (CH, 16), 186.4 (C, OAc), 208.5 (C), 210.5 (C), 211.0 (C); LR-MS: ES$^+$ [M+Na]$^+$: 411 (100%); HR-MS: ES$^+$ [M+Na]$^+$; calc for C$_{23}$H$_{32}$O$_5$Na = 411.2142 found 411.2159; [$\alpha$]$_D^{20}$ = -5.0° (c = 0.5, MeOH).

24,25-Dihydro fusidic acid; 29-Nor-8α,9β,13α,14β-dammar-17(20)-en-21-oic acid, 3α,11α,16β-trihydroxy-, 16-acetate, (Z)- (8CI) (250)

![Diagram of fusidic acid and reaction](image)

Fusidic acid 166 (10.6 mg, 20.5 µmol), 5 wt% palladium on an activated charcoal catalyst (0.1 mg, 9.4 µmol) and ethanol (1 mL) was stirred for 24 hrs under a H$_2$ atmosphere. The palladium catalyst was then filtered off and washed with ethanol (5 mL), before the solvent was then reduced to dryness in vacuo to afford 250 (6.3 mg, 59.0% yield) as a white solid.69
$^1$H NMR: (400 Hz, CDCl$_3$), $\delta = 0.79$ (d, 6H, $J = 6.7$ Hz, 26, 27), 0.83-0.87 (m, 6H, 18, 28), 0.91 (s, 3H, 19), 0.95-1.15 (m, 2H, 7a, 6a), 1.21 (dd, 1H, $J = 14.2$, 6.7 Hz, 15a, 23), 1.31 (s, 3H, 29), 1.35-1.6 (m, 1a, 9, 4, 6b, 25), 1.63-1.72 (m, 2H, 7b, 2a), 1.73-1.85 (m, 2H, 12a, 2b), 1.91 (s, 3H, OAc), 1.95-2.15 (m, 4H, 1b, 5, 24), 2.22 (dt, 1H, $J = 13.1$, 2.5 15b), 2.31-2.45 (m, 2H, 12b, 22), 2.98 (d, 1H, $J = 11.2$ Hz, 13), 3.70 (bd, 1H, $J = 2.5$ Hz, 3), 4.28 (bd, 1H, $J = 1.7$ Hz, 11), 5.80 (d, 1H, $J = 8.4$ Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta = 14.9$ (CH$_3$, 28), 17.8 (CH$_3$, 18), 19.6 (CH$_3$, Ac), 19.7 (CH$_2$, 6), 21.5 (CH$_3$, 26, 27), 21.6 (CH$_2$, 24), 21.7 (CH$_3$, 19), 23.2 (CH$_3$, 29), 26.7 (CH$_2$, 23), 26.8 (CH, 25), 27.9 (CH$_2$, 22), 28.9 (CH$_2$, 2), 29.3 (CH$_2$, 1), 31.5 (CH$_2$, 7), 34.4 (CH$_2$, 12), 35.1 (CH, 5), 35.2 (CH, 4), 36.1 (C, 10), 38.0 (CH$_2$, 15), 38.4 (C, 8), 43.1 (CH, 13), 47.7 (C, 14), 48.2 (CH, 9), 67.3 (CH, 11), 70.4 (CH, 3), 73.4 (CH, 16), 128.8 (C, 20), 149.1 (C, 17), 169.6 (C=O, Ac), 172.0 (C, 21); LRMS: ES$^+$ [M-H]: 517 (100%), [M+Cl$^-$]: 551 (10%); HR-MS: ES$^+$ [M-H]: calc for C$_{31}$H$_{49}$O$_6$ equals 517.3534 found 517.3535; IR (powder) $v_{\text{max}}$: 3404, 2952, 2923, 2870, 1712, 1692, 1467, 1375, 1260, 1120, 971, 520 cm$^{-1}$. $[\alpha]_b^{20} = -3.2^\circ$ (c =1, CHCl$_3$), Lit = +/-0°.226
24,25-Oxidofusidic acid; 29-Nor-8α,9β,13α,14β-dammar-17(20)-en-21-oic acid, 24,25-epoxy-3α,11α,16β-trihydroxy-, 16-acetate, (E)- (8CI), (252) and (253)

Reaction performed as described by Porto et al. Fusidic acid (30.1 mg, 58.3 µmol) was dissolved in dichloromethane (0.5 mL), before m-chloroperbenzoic acid (12.1 mg, 70.0 µmol) was added at 0 °C. The solution was stirred for 24 hrs, before being washed with a 10% KOH solution (1.5 mL). The product was then extracted with dichloromethane (3 x 1.5 mL), dried with MgSO₄ and then reduced to dryness in vacuo. This afforded a mix of stereo-isomers 252 and 253. These isomers were separated via reverse phase HPLC (Licrosorb RP-18 10µm 25 x 0.78 column, 3 ml/min, 80:20 MeOH:H₂O solvent system), affording product 1, 8.77 min (2.3 mg, yield = 7.4%) and product 2, 10.16 min (25.8 mg, yield = 83.1%), both had the appearance of white powders.

Product 1

¹H NMR: (400 Hz, CDCl₃), δ = 0.84-0.88 (m, 6H), 0.91 (s, 3H), 0.95-1.15 (m, 4H), 1.16-1.22 (m, 6H), 1.31 (s, 3H), 1.36-1.74 (m, 11H), 1.96 (s, 3H), 1.94-2.22 (m, 2H), 2.2-2.61 (m, 4H), 2.85-3.15 (m, 2H), 3.44 (bd, 1H, J = 10.9 Hz), 3.70 (dd, 1H, J = 2.0, 10.3 Hz), 3.93 (dd, 1H, J = 2.8, 11.3 Hz), 4.29 (bs, 1H), 5.82 (d, 1H, J = 6.8 Hz); LR-MS: ES⁺ [M+Na]⁺: 555 (100%), HR-MS: ES⁺ [M-H]⁻: calc for C₃₁H₄₈O₇Na 555.3293 found 555.3274.
Product 2

$^1$H NMR: (400 Hz, CDCl$_3$), $\delta$ = 0.84-0.88 (m, 6H, 18, 28), 0.91 (s, 3H,19), 0.94-1.14 (m, 1H, 6a), 1.18 (s, 6H, 26, 27), 1.20-1.28 (m, 2H, 15a, 22), 1.31 (s, 3H, 29), 1.36-1.74 (m, 10H, 1a, 2a, 6b, 7a, 7b, 12a, 15b, 23, 4, 9), 1.80 (m, 1H, 2b), 1.92 (s, 3H, OAc), 1.94-2.22 (m, 2H, 1b, 5), 2.28-2.35 (m, 1H,12b), 3.06 (dd, 1H, J = 2.7, 12.1 Hz, 13), 3.70 (bd, 1H, J = 2.5 Hz, 3), 3.89 (dd, 1H, J = 1.9, 11.5 Hz, 24), 4.29 (d, 1H, J = 1.8 Hz, 11), 5.97 (d, 1H, J = 8.7 Hz, 16); $^{13}$C NMR: (400 Hz, CDCl$_3$), $\delta$ = 16.0 (CH$_3$, 28), 17.0 (CH$_3$, 18), 18.1 (CH$_3$, 19), 19.4 (CH$_2$, 6), 20.0 (CH$_3$, OAc), 21.7 (CH$_2$, 22), 23.5 (CH$_3$, 29), 28.6 (2xCH$_3$, 26, 27), 28.9 (CH$_2$, 2), 29.4 (CH$_2$, 1), 30.9 (CH$_2$, 23), 31.7 (CH$_2$, 7), 33.3 (CH, 4), 34.6 (CH$_2$, 12), 35.4 (CH, 5), 37.2 (C, 10), 38.3 (CH$_2$, 15), 45.4 (CH, 13), 48.7 (C, 14), 47.9 (CH, 9), 67.5 (CH, 11), 70.5 (CH, 3), 73.7 (CH, 16), 83.8 (CH, 24), 90.6 (C, 25), 122.7 (C, 20), 149.6 (C, 17), 170.4 (C, OAc), 175.2 (C, 21); LR-MS: ES$^+$ [M+Na]$^+$: 555 (100%); HR-MS: ES$^+$ [M-H]$^-$: calc for C$_{31}$H$_{48}$O$_7$Na 555.3293 found 555.3300; FTIR (powder) $\nu_{\max}$: 3399, 2933, 2870, 1601, 1530, 1452, 1404, 1318, 1251, 1217, 1058, 755, 712, 472 cm$^{-1}$; $[\alpha]_D^{20}$ = +15.5° (c = 0.25, MeOH).

trans-1-bromo but-2-ene-4-thioacetate (256) and trans-2-butene-1,4-dithioacetate (274)

trans-1,4-dibromo but-2-ene 273 (2.5 g, 11.70 mmol) was dissolved in DMF (20 mL), before potassium thioacetate (1.34 g, 11.70 mmol) in DMF (10 mL) was added drop wise. The reaction was stirred for 24 hrs, before the solution was diluted with 1N HCl (10
mL), extracted with Et₂O (3 x 25 mL), washed with brine (25 mL) and then dried with MgSO₄ before being reduced to dryness in vacuo. Purification via silica chromatography (40:1 hexane:ethyl acetate) gave trans-1-bromo-2-butene-4-thioacetate 256 (1.10 g, yield = 45%) and trans-2-butene-1,4-dithioacetate 274 (0.79 g, yield = 33%). Both had the appearance of colourless oils.²³⁴

²⁳⁴¹H NMR: (400 Hz, CDCl₃), δ = 2.27 (s, 3H, 6), 3.47 (d, 2H, J = 6.3 Hz, 4), 3.85 (dd, 2H, J = 7.3, 0.5 Hz, 1), 5.6-5.75 (m, 1H, 3), 5.74-5.9 (m, 1H, 2); ¹³C NMR: (300 Hz, CDCl₃), δ = 29.4 (CH₂, 4), 29.5 (CH₃, 6), 30.7 (CH₂, 1), 128.6 (CH, 3), 129.2 (CH, 2), 193.7 (C=O, 5).

²³⁴¹H NMR: (400 Hz, CDCl₃) δ = 2.26 (s, 3H, 4), 3.42 (dd, 2H, J = 1.8, 4.0 Hz, 2), 5.57 (ddd, 1H, J = 1.8, 4.0, 5.7 Hz, 1); ¹³C NMR: (300 Hz, CDCl₃) δ = 30.4 (CH₂, 4), 30.7 (CH₂, 2), 128.4 (CH, 1), 194.7 (C=O, 3).

2-(Acethylthio)acetic acid (254)

Mercaptoacetic acid 275 (1.51 g, 15.56 mmol), 4-Dimethylaminopyridine (10 mg, 8.20 μmol) and acetic anhydride (1.91 g, 18.67 mmol) was dissolved in acetonitrile (10 mL). Triethylamine (2.1 mL, 31.12 mmol) was added drop wise at 0 °C and the reaction was stirred for 24 hrs. The solution was then diluted with 1N HCl (5 mL), extracted with EtOAc (3 x 10 mL), washed with brine (10 mL) and then dried with MgSO₄. The solution was reduced to dryness in vacuo to afford a crude yellow oil. Purification via silica chromatography (40:1 hexane:ethyl acetate) afforded 254 (1.40 g, yield = 67.0%) as a pale yellow oil.²³⁵
1H NMR: (400 Hz, CDCl₃), δ = 2.34 (s, 3H, 4), 3.68 (s, 1H, 2); 13C NMR: (300 Hz, CDCl₃), δ = 31.4 (CH₃, 1), 39.0 (CH₂, 2), 174.1 (C, 3), 194.1 (C, 1).

Fusidic acid-21-methyl ester, 3-acetate-31-(acetythio) (255)

Methyl fusidic acid 221 (9.9 mg, 18.7 µmol) was reacted with mercaptoacetic acid acetate (3.0 mg, 26.2 µmol) using the general esterification method, which afforded 255 (10.9 mg, 92.3% yield) as a white solid.

1H NMR: (400 Hz, CDCl₃), δ = 0.83 (s, 3H, 28), 0.85 (d, 3H, J = 6.9 Hz, 18), 0.90 (s, 3H, 19), 0.94-1.12 (m, 3H, 2a, 6a, 7a), 1.20-1.24 (m, 1H, 15a), 1.30 (s, 3H, 29), 1.30-1.48 (m, 2H, 1a, 6b), 1.49 (d, 1H, J = 1.5 Hz, 9), 1.52 (s, 3H, 26), 1.60 (s, 3H, 27), 1.63-1.83 (m, 4H, 2b, 5, 7b, 12a), 1.91 (s, 3H, OAc), 1.93-2.15 (m, 8H, 1b, 4, 15b, 23a, 23b, SAc), 2.25 (dt, 1H, J = 3.2, 13.1 Hz, 12b), 2.29-2.47 (m, 2H, 22), 2.96 (d, 1H, J = 10.8 Hz, 13), 3.57 (s, 3H CO₂Me), 4.27 (bd, 1H, J = 1.8 Hz, 11), 4.54 (bd, 1H, J = 2.6 Hz, 3), 5.02 (tt,
1H, \( J = 7.2, 1.2 \text{ Hz, 24} \), 5.77 (d, 1H, \( J = 8.4 \text{ Hz, 16} \)); \(^{13}C\) NMR: (400 Hz, CDCl\(_3\)), \( \delta = 15.8 \) (CH\(_3\), 28), 16.6 (CH\(_3\), 26), 17.0 (CH\(_3\), 18), 20.7 (CH\(_3\), OAc), 20.8 (CH\(_2\), OAc), 21.0 (CH\(_3\), 19), 24.1 (CH\(_3\), 29), 25.8 (CH\(_3\), 27), 26.3 (CH\(_2\), 2), 28.3 (CH\(_2\), 23), 28.9 (CH\(_2\), 22), 30.2 (CH\(_2\), 1), 32.3 (CH\(_2\), 7), 35.5 (CH\(_2\), 12), 36.2 (CH, 4), 37.0 (C, 10), 38.2 (CH, 5), 38.7 (CH\(_2\), 31), 39.0 (CH\(_2\), 15), 39.4 (C, 8), 43.9 (CH, 13), 48.7 (C, 14), 49.2 (CH, 9), 51.4 (CH\(_3\), CO\(_2\)Me), 68.3 (CH, 11), 71.4 (CH, 3), 74.4 (CH, 16), 123.1 (CH, 24), 130.4 (C, 20), 132.6 (C, 25), 148.2 (C, 17), 170.4 (C=O, 21), 170.8 (C=O, OAc), 171.5 (C=O, 30).

16-Deacetyl fusidic acid; 29-Nor-8\( \alpha\),9\( \beta\),13\( \alpha\),14\( \beta\)-dammara-17(20),24-dien-21-oic acid, 3\( \alpha\),11\( \alpha\),16\( \beta\)-trihydroxy-, (Z)-(8CI) (258)

Fusidic acid 168 (50.0 mg, 0.0928 mmol) was dissolved in 10% NaOH/EtOH solution (0.5 ml) and stirred for 24 hours. The reaction was quenched with 2N HCl (0.5 mL), extracted with ethyl acetate (3 x 1.5 mL) and dried with MgSO\(_4\). The solution was then evaporated to dryness in vacuo, affording 258 (40.7 mg, yield = 92.5%) as a white solid.\(^{236}\)
1H NMR: (400 Hz, CDCl₃), δ = 0.77 (m, 6H, 18, 28), 0.82 (s, 3H, 19), 0.85-1.20 (m, 2H, 6a, 7a) 1.25 (s, 3H,29), 1.30-1.45 (m, 4H, 2a, 4, 6b, 9), 1.49 (s, 3H, 26), 1.55 (s, 3H, 27), 1.57-1.90 (m, 9H, 1a, 1b, 7b, 12a, 12b, 15a, 15b, 23), 1.95-2.15 (m, 3H, 2b, 5, 22a), 2.17-2.35 (m, 1H, 22b), 2.92 (dd, 1H, J = 3.2, 13.2 Hz, 13), 3.62 (d, 1H, J = 3.6 Hz, 3) 4.18 (bd, 1H, J = 1.4 Hz, 11), 4.55 (d, 1H, J = 6.6 Hz, 16), 4.93 (t, 1H, J = 6.9 Hz, 24); 13C NMR: (400 Hz, CDCl₃), δ = 15.1 (CH₃, 28), 17.8 (CH₃, 26), 19.1 (CH₃, 18), 20.9 (CH₂, 6), 21.5 (CH₂, 22), 22.8 (CH₃, 19), 24.3 (CH₃, 29), 25.7 (CH₃, 27), 29.6 (CH₂, 23), 30.3 (CH₂, 2), 30.9 (CH₂, 1), 32.8 (CH₂, 7), 33.2 (CH₂, 12), 36.1 (CH, 5), 36.2 (CH, 4), 37.1 (C, 10), 39.0 (CH₂, 15), 41.1 (C, 8), 43.6 (CH, 13), 49.1 (C, 14), 49.3 (CH, 9), 68.2 (CH, 11), 71.5 (CH, 3), 89.5 (CH, 16), 123.4 (CH, 24), 128.2 (C, 20), 133.4 (C, 25), 148.2 (C, 17), 176.6 (C, 21); FTIR (powder): 3433, 2928, 2870, 1731, 1692, 1443, 1376, 1251, 1029, 977, 933, 530 cm⁻¹; [α]D²⁰ = -44.6° (c =1, CHCl₃), Lit = -57°.²²⁶

Deacetyl-16-epifusidic acid γ-lactone; 29-Nordammara-17(20),24-dien-21-oic acid, 3,11,16-trihydroxy-, γ-lactone, (3α,4α,8α,9β,11α,13α,14β,16α)-(9CI) (259)

Fusidic acid 168 (20.1 mg, 38.9 µmol) was dissolved in a 1:1 EtOH:aqNaOH (2M, 0.5 mL) mixture, before being heated to 80 °C for 4 hours. The solution was then diluted with H₂O (1mL), extracted with EtOAc (3 x 1.5 mL) and evaporated to dryness in vacuo, which afforded lactone 259 (15.7 mg, yield = 88.2%) as a white powder. 1H and 13C NMR were equivalent to literature assignments, published by Rastrup-Andersen et al.⁶⁹
$^1$H NMR: (400 Hz, MeOD), δ = 0.72 (s, 3H, 18), 0.81 (d, 3H, $J$ = 6.8 Hz, 28), 0.89 (d, 3H, 19), 1.00-1.14 (m, H, 6a, 7a, 15a), 1.40 (dt, 1H, $J$ = 13.7, 3.3 Hz, 4), 1.46 (m, 3H, 29, 9), 1.53 (s, 3H, 26), 1.54-1.59 (m, H, 6b, 7b), 1.61 (s, 3H, 27), 1.62-1.77 (m, H, 2a, 2b, 1a), 1.79 (dd, 1H, $J$ = 12.9, 2.5 Hz, 12a), 1.83-2.03 (m, H, 12b), 2.03-2.20 (m, 4H, 5, 22, 23a), 2.20-2.37 (m, 3H, 1b, 15b, 23b), 3.55 (dd, 1H, $J$ = 12.8, 2.9 Hz, 13), 3.59 (bd, 1H, $J$ = 3.6 Hz, 3), 4.26 (bd, 1H, $J$ = 2.2 Hz, 11), 4.99 (dd, 1H, $J$ = 10.9, 4.3 Hz, 16), 5.07 (t, 1H, $J$ = 5.9 Hz, 24); $^{13}$C NMR: (400 Hz, MeOD), δ = 16.6 (CH$_3$, 28), 20.4 (CH$_3$, 26), 22.6 (CH$_2$, 6), 23.0 (CH$_3$, 18), 24.2 (CH$_3$, 29), 24.3 (CH$_3$, 19), 24.9 (CH$_2$, 23), 26.0 (CH$_3$, 27), 28.4 (CH$_2$, 22), 30.8 (CH$_2$, 2), 31.0 (CH$_2$, 1), 32.3 (CH$_2$, 7), 33.3 (CH$_2$, 12), 34.9 (CH$_2$, 15), 36.8 (CH, 5), 38.0 (C, 10), 38.5 (CH, 4), 39.6 (CH, 13), 42.1 (C, 8), 51.8 (C, 9), 56.7 (C, 14), 68.1 (CH, 11), 72.4 (CH, 3), 83.9 (CH, 16), 124.1 (C, 20), 124.4 (CH, 24), 133.8 (C, 25), 173.3 (C, 17), 179.0 (C, 21); LRMS: ES$^+$ [M+Na]$^+$: 479 (64%); HR-MS: ES$^+$ [M-H]$^-$: calc for C$_{20}$H$_{44}$O$_4$Na 479.3132 found 479.3135; $[a]_D^{20}$ = +60.2° (c = 0.25, CHCl$_3$), Lit = +58°.226
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