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Application of an affinity chromatography toolbox to drug repurposing for cancer therapeutics

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Declaration

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at The University of Edinburgh. Unless otherwise stated, the work described in this thesis is original and has not been submitted previously in whole or in part for any degree or other qualification at this or any other university. In accordance with the dissertation regulations as specified by The University of Edinburgh, this thesis does not exceed 100,000 words in length.

Faye Cruickshank
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"The best laid schemes o' mice an' men, gang aft agley" – Robert Burns
Abstract

Phenotypic screening of drug molecules relies on the generation of a specific response; however the means by which this is elicited often remains unknown. Affinity chromatography is a valuable tool in the discovery of drug binding partners and may even allow the elucidation of the wider interactome of the initial drug target. The introduction of easily cleavable linkers and affinity-independent elution protocols to affinity chromatography is of current interest, since they render the technique much more adaptable with respect to the characterisation of biologically active species of interest. This thesis details the application of a novel azobenzene linker developed by the Hulme group for use in affinity-independent chromatography.

The first chapter reviews recent developments in affinity chromatography and describes the synthesis of an affinity linker toolbox with both affinity-dependent and affinity-independent linkers. These linkers are functionalised with an azide moiety for use in CuAAC coupling to alkynyl derivatives of bioactive small molecules and have been modified to include photoreactive groups giving a series of linkers for use in the identification of less abundant, or low affinity, proteins.

The first drug investigated, anisomycin (ANS), is a small molecule which was initially introduced as an antibiotic drug (Flagecidin). At nanomolar concentrations ANS has been shown to affect the mitogen activated protein kinase (MAPK) pathways; downstream effects of these pathways are thought to play a role in a range of pathological disorders such as Alzheimer’s disease, cancer and spinal muscular atrophy (SMA). ANS is thus a candidate for drug repurposing. Although the
downstream effects of MAPK/SAPK pathway activation induced by anisomycin are well-documented, the cellular target has yet to be revealed. Previous work by the Hulme group has shown that the $N$-propargyl anisomycin derivative (I) retains the biological activity of the lead compound ANS. Thus to evaluate the cellular protein targets, $N$-propargyl ANS (I) was coupled onto the linker toolbox to create an ANS affinity probe library as described in chapter 2.

The second drug investigated, fingolimod, was introduced as an immunomodulating drug (Glienya) for the treatment of multiple sclerosis (MS). This small molecule has also been shown to have anti-cancer properties in a range of cancer cell lines; however the precise mechanism by which this is effected is unknown. Literature precedent shows that terminal modification of fingolimod generates analogues which still retain biological activity. Thus a novel fingolimod alkyne derivative (II) was synthesised and used to create an affinity probe library as described in chapter 3.

Chapter 4 describes affinity pull-down experiments conducted with the aim of finding the protein target(s) of ANS and fingolimod, using the affinity probe libraries generated in chapters 2 and 3. This chapter concludes with a discussion of the implications of these findings and directions for future study.
Lay Summary

One method for screening drug molecules relies on the generation of a desired effect, or response, in the biological system under study. However, a major drawback of this approach is that the precise mechanism by which this is achieved is often not well understood. Many proteins have the capacity to bind specific molecules tightly but reversibly; affinity chromatography can utilise these interactions to give a clearer picture of biological targets by their separation from complex mixtures. Affinity chromatography can be applied to the discovery of drug-protein binding interactions, thus allowing the elucidation of a specific biological response pathway. This is frequently referred to as ‘fishing’ for the molecular target of a drug.

The introduction of easily cleavable linkers and affinity independent elution protocols to affinity chromatography is of current interest, since these adaptations render the technique much more adaptable with respect to the characterisation of biologically active species.

This thesis details the synthesis of a series of molecular ‘fishing rods’ to which a bioactive small molecule ‘bait’ can be attached, for use in affinity chromatography. The ‘bait’ are two known drug molecules, anisomycin and fingolimod, both of which have been shown to have anti-cancer effects in several cancer cell lines, though their precise mechanisms of action are yet to be fully characterised.
Contents

Title Page ................................................................. I
Declaration ....................................................................... II
Acknowledgements ........................................................... III
Abstract ........................................................................ IV
Lay Summary .................................................................... VI

Chapter 1  Affinity Chromatography .................................... 1

1.1  Introduction ................................................................ 1

1.2  Theory .................................................................. 1

1.3  Biomolecular interactions .............................................. 2

1.4  Central components of affinity chromatography ............... 4

1.4.1  The matrix .......................................................... 4

Agarose and other porous resins ............................................. 5
Magnetic particles ................................................................. 5
Noncovalent immobilisation .................................................... 6

1.4.2  Spacer Arm ............................................................ 7

1.4.3  The eluent ............................................................. 8

1.4.4  The Ligand ............................................................. 9

Non-covalent interactions ...................................................... 11
Affinity-based protein profiling .............................................. 11
Covalent interactions .......................................................... 13
Activity based protein profiling (AcBPP) ................................ 13
Photoaffinity labelling .......................................................... 15

1.5  Affinity independent elution ......................................... 21

1.5.1  Enzymatic cleavage ............................................... 21

1.5.2  Photolytic cleavage ................................................ 23

o-Nitrobenzyl derivatives .................................................... 23
Novel photocleavable linker ................................................... 25

1.5.3  Chemical cleavage .................................................. 26

Disulfide bond reduction cleavage ......................................... 26
Azobenzene linkers ............................................................. 26
Chapter 2 Anisomycin ................................................................. 39
  2.1 Introduction ........................................................................ 39
    2.1.1 Natural products in drug discovery ................................. 39
    2.1.2 Drug repurposing .......................................................... 39
    2.1.3 Anisomycin ................................................................. 41
    2.1.4 Previous biological work ................................................ 44
  2.2 Results ............................................................................... 47
    2.2.1 Anisomycin affinity probes ............................................. 47
  2.3 Discussion ........................................................................... 49
Chapter 3 Fingolimod ................................................................. 51
  3.1 Introduction ........................................................................ 51
    3.1.1 Sphingolipids ............................................................... 51
    3.1.2 Fingolimod ................................................................. 52
      Introduction ......................................................................... 52
      Biological mode of action – immunomodulation .................... 53
      Fingolimod and multiple sclerosis .......................................... 56
      Fingolimod and cancer ....................................................... 57
  3.2 Results ............................................................................... 59
    3.2.1 Literature precedent ...................................................... 59
    3.2.2 Fingolimod analogue synthesis ...................................... 60
    3.2.3 Polar head group synthesis ........................................... 61
      Aryl bromide ..................................................................... 61
      Aryl iodide ........................................................................ 62
    3.2.4 Sonogashira synthesis of fingolimod alkyne ...................... 63
    3.2.5 Zipper /Negishi synthesis of fingolimod alkyne ............... 66
      Summary - Approaches to fingolimod alkyne ......................... 67
3.2.6 Affinity probe synthesis ................................................................. 67
3.3 Discussion .......................................................................................... 69

Chapter 4 Biological Studies ...................................................................... 70
4.1 Anisomycin .......................................................................................... 70
  4.1.1 Ribosomal Binding Study ................................................................. 70
  4.1.2 Affinity Pull-Down Experiments ..................................................... 75
4.2 Fingolimod ......................................................................................... 77
  4.2.1 Preliminary pull down experiments ............................................... 77
  4.2.2 Western Blot ................................................................................. 85
  4.2.3 Conclusion ................................................................................... 89
    Affinity-dependent fingolimod probes ................................................ 89
    Affinity-independent fingolimod probes ........................................... 90
4.3 Future Work ....................................................................................... 91
  4.3.1 Anisomycin .................................................................................. 91
  4.3.2 Fingolimod .................................................................................. 91

Chapter 5 Experimental ............................................................................. 92
5.1 General Experimental ......................................................................... 92
5.2 Experimental for Chapter 1 ................................................................. 94
5.3 Experimental for Chapter 2 ................................................................. 119
5.4 Experimental for Chapter 3 ................................................................. 127
5.5 Experimental for Chapter 4 ................................................................. 148

Chapter 6 References ................................................................................. 156
Chapter 1  Affinity Chromatography

1.1 Introduction

A distinguishing feature of many proteins is their capacity to bind specific molecules tightly but noncovalently. This feature allowed for the development of affinity chromatography, and since the early 1950’s it has become one of the most valuable techniques available for the isolation of biomolecules. Unlike other chromatographic methods, such as ion exchange/gel filtration chromatography, which use small variances in the physicochemical properties of the proteins, this method exploits the desired protein’s unique biochemical properties. This creates a greater separation power than any other chromatographic technique. This seminal tool in the biochemistry field of research has expanded recently due to the introduction of new and improved solid supports, materials, equipment and the expansion of the technique from a research tool to a widely used, industrial scale biopurification method.

1.2 Theory

Affinity chromatography is a method by which one or more components of a biochemical mixture can be purified on the basis of “biorecognition”. A biospecific or chemically specific interaction between a protein and ligand which is coupled to the affinity chromatography matrix creates a reversible adsorption interaction (Figure 1.1). This technique can be used whenever a suitable ligand is available for the biomolecule of interest, and is ideal for a capture or intermediate step in a purification protocol. The high selectivity of affinity chromatography enables many separations to be achieved in one simple step, including the purification of monoclonal antibodies or fusion proteins. The target biomolecule binding to the ligand can be due to electrostatic or hydrophobic interactions, Van der Waals forces and/or hydrogen bonding. In order to recover the biomolecule from the affinity matrix, these
interactions must be reversed. The elution can be carried out either specifically; (using a competitive ligand), or non-specifically; (by altering the pH, ionic strength or the polarity of the eluent mobile phase).

**Figure 1.1** – Affinity chromatography purification scheme. (a) Sample mixture is applied under conditions that will favour binding between ligand and biomolecule. (b) Target binds reversibly and specifically to the ligand, and unbound material is washed away. (c) Recovery of the target biomolecule using elution to disrupt interactions. (d) The affinity solid support is recovered by equilibration with binding buffer.

For a successful application, the coupled ligand must retain its specific binding affinity for the target molecule and, once the unbound material has been removed by washing, the binding between the ligand and the target biomolecule must be reversible to allow the target to be eluted in its active, non-denatured form.\(^2\)\(^,\)\(^6\) Many known biological interactions are utilised in the field of affinity chromatography, such as; enzyme/substrate (or inhibitor), antibody/antigen (or virus), lectins/carbohydrates (or glycoprotein) etc.\(^5\)

### 1.3 Biomolecular interactions

The biological interactions that occur between the target molecule and the ligand involve an array of typically non-covalent contacts, whereby the forces involved may be complementary and additive. Thus the total of the forces determines the
dissociation constant \((K_d)\), where A is a protein, B is a ligand and AB is the complex formed between the two species (Figure 1.2).

\[
K_d = \frac{[A][B]}{[AB]}
\]

**Figure 1.2** – a) Illustration of binding complex; A is the protein, B is the ligand, and AB is the complex formed; and b) the calculation of the dissociation constant \((K_d)\).

Both the chemical and the mechanical properties of the affinity solid support are significant to the outcome of the experiment, as interactions that occur during the procedure can vary the binding affinity \((K_d)\) to the solid support. Problems with binding affinity, such as non-specific interactions between the solid phase and the ligand and steric hindrance of the ligand due to the solid phase, have been resolved practically by the insertion of inert spacer arms.\(^7\)\(^8\)
1.4 Central components of affinity chromatography

The four main components of an affinity chromatography system, as illustrated in Figure 1.3, are; the matrix (stationary phase), a spacer arm, a ligand (one of the two members of an interacting pair), and the eluent buffer.

Figure 1.3 – Physical components of an affinity solid support. (a) Matrix support designed for ligand attachment; must be chemically and physically inert. (b) Spacer arm is designed to improve binding between ligand and target. (c) Ligand to which the target binds. (d) Eluent which runs through the column reverses interaction between bound material and ligand.

1.4.1 The matrix

The purpose of the matrix is for ligand attachment, and so it is critical for the solid support to be a chemically and physically inert material to which the ligand can be directly or indirectly coupled. Many types of affinity matrices exist, made from agarose, glass, cellulose, dextrans etc., all with differing chemical and physical properties to allow for different applications. The efficiency of affinity chromatography relies on specific biological interactions, and so a particularly low non-specific adsorption for the matrix leads to a more successful procedure. Other attributes essential for an affinity matrix are: an open pore structure, allowing high capacity binding and larger biomolecules to flow efficiently; good flow properties to enhance separation; insolubility in solvents and buffers employed in the process; and stability of the matrix under a range of different experimental conditions, (e.g. pH, detergents, and dissociating agents).
Affinity Chromatography

Agarose and other porous resins

Agarose has been widely used as a stationary phase for affinity chromatography, due to its favourable physical and chemical properties, such as: high chemical stability, hydrophilicity, high exclusion limit and low tendency for non-specific interactions.\(^\text{10}\)

\[\text{Figure 1.4 – a) The agarose monomeric subunit, b) aggregation of the polymer agarose gives a porous structure, and c) the dextran monomeric subunit.}\]

The agarose has hydroxyl groups on the sugar residues which are readily derivatised for ligand attachment (Figure 1.4, a), providing an ideal media for affinity chromatography.\(^\text{10}\) Sepharose is the bead form of agarose, and has been modified and developed further to enhance its aggregation properties, (e.g. cross-linking to increase stability, and differing particle size and porosity to maximise ligand binding sites).\(^\text{9,11}\)

Dextran, a glucose polymer (Figure 1.4, c) may be cross-linked to give Sephadex, which is stable within a pH range of 2-10 in most aqueous and organic eluent systems. The gel filtration matrix Superdex is a composite of dextran covalently attached to highly cross-linked agarose. This solid support shows excellent high resolution fractionation of biomolecules with high recovery, and is very stable (tolerates repeated autoclaving, high pressure and exposure to abrasive chemicals).\(^\text{10}\)

Magnetic particles

Biocompatible magnetic particles are not a new discovery in chemistry,\(^\text{12,13}\) however the use of magnetic particle beads is a recent discovery in affinity chromatography, being used for ‘ligand fishing’,\(^\text{14}\) and further developed since.\(^\text{15}\) Magnetic particles are
a completely different type of affinity support from beaded agarose and other porous resins. They are much smaller and non-porous. Their small size provides the required surface area-to-volume ratio needed for effective ligand immobilization and affinity purification. Magnetic beads are produced as superparamagnetic iron oxide particles that are covalently coated with a thin polymer shell.\textsuperscript{16} The coating makes the beads inert to minimize nonspecific binding and provides the particular chemical groups needed for attaching ligands of interest. The particular advantage of magnetic particles over porous resins is their suitability for high-throughput automation. Another advantage of magnetic particles is that, unlike porous resins, they can be used in immunomagnetic cell separation.\textsuperscript{17}

**Noncovalent immobilisation**

Several biologically mediated immobilisation techniques exist which offer an alternative approach to covalent immobilisation of the affinity ligand on the solid support, such as polyhistidine tags, antibody-based, and glutathione-GSH-based immobilisation.\textsuperscript{18,19}

The most popular and extensively researched immobilisation method is the biotin-(strept)avidin interaction. The interaction, first found by Eakin \textit{et al}.\textsuperscript{20} and utilised biochemically by Cuatrecasas \textit{et al}.\textsuperscript{21} is one of the strongest noncovalent binding affinities known ($K_d \approx 1 \times 10^{-15}$ M) in nature.

The biological properties of the two proteins avidin (Av) and streptavidin [(S)Av] are very similar (Table 1.1), the main difference being that due to avidin’s high pI and carbohydrate content, it binds non-specifically to components other than biotin.
### Table 1.1 – Comparison of Av and (S)Av

<table>
<thead>
<tr>
<th></th>
<th>Avidin</th>
<th>Streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isolation</strong></td>
<td>Egg-whites, glycoprotein</td>
<td>Streptomyces avidinii</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>Tetramer, 86 kDa</td>
<td>Tetramer, 60 kDa</td>
</tr>
<tr>
<td><strong>Isoelectric point (pI)</strong></td>
<td>10</td>
<td>5 – 6</td>
</tr>
<tr>
<td><strong>Dissociation constant</strong></td>
<td>$10^{-15}$ M</td>
<td>$10^{-12}$ M</td>
</tr>
<tr>
<td><strong>Target(s)</strong></td>
<td>4 x biotin, 4 x oligosaccharide</td>
<td>4 x biotin</td>
</tr>
</tbody>
</table>

The strength of the noncovalent binding interaction, as well as the robustness of the proteins to tolerate a wide range of pH, buffers and chemical modification, without loss of binding affinity, renders this technique valuable for bioconjugate chemistry.

In the scope of affinity chromatography, the tight binding of (S)Av to biotin allows biotin-linked ligands in complex mixtures to be discretely bound to solid supported (S)Av.

### 1.4.2 Spacer Arm

Success of affinity chromatography resides in the ability of the affinity matrix to bind to the target protein, therefore if the target protein recognition site cannot bind with the immobilized ligand the affinity technique is essentially useless. Binding sites for target proteins are frequently buried deep within the biomolecule, and an affinity solid support with a small ligand attached, such as an enzyme co-factor, may show low binding affinity due to steric interference of the solid support and thus inefficient ligand binding (Figure 1.5).
Figure 1.5 – The importance of the correct spacer arm length in ensuring optimum binding, a) shows optimum spacer length (maximum binding), b) ineffective short spacer length, and binding affinity reduced.

A spacer arm is used to maximise binding between ligand and target biomolecule by overcoming any steric factors that can occur. The spacer arm length is subject to the ‘Goldilocks effect’ and is critical: too short and the arm is ineffective, too long and the number of non-specific interactions between the protein and the spacer arm increase, and so a balance between these two effects must be sought. Spacer arms should neither chemically nor structurally affect the target biomolecule or the ligand.

1.4.3 The eluent

Retrieval of target biomolecules from the affinity matrix is a crucial step in affinity experiments, where proteins isolated are in either their native or non-native state. The choice of elution protocol is often dependent on the protein-ligand interaction and whether the mode of binding is known or not. The elution buffer is able to dissociate binding partners through extremes of pH (low or high), high salt (ionic strength), the use of detergents or chaotropic agents that denature one or both of the molecules, removal of a binding factor, or competition with a counter ligand for either the target or the ligand (Figure 1.6). The ‘black art’ of target elution from affinity chromatography sometimes requires a trial and error approach when the exact binding interaction is not known.
Figure 1.6 – Comparison of elution techniques. (1) A change in buffer composition elutes the bound substance without harming it or the ligand. (2) pH extremes or high concentrations of chaotropes are required for elution, and may cause permanent or temporary damage. (3) Specific elution by using a competitor for binding to the ligand. (4) Specific elution using a competitor which binds to the target.

1.4.4 The Ligand

Another species present on the solid support is the ligand, which is a molecule that binds to the target biomolecule(s) to allow affinity chromatography to occur. The choice of ligand is dependent on the target, as it must show specific binding affinity, and have chemically modifiable groups to allow attachment onto the solid support without losing any binding affinity. One of the principle difficulties when attempting to couple the ligand to the spacer arm or matrix is consideration of the structure activity relationship (SAR) between the affinity tag and the target biomolecule. The ligand must be bound in a way that will not be detrimental to the activity of the molecule. Modification of the ligand begins with the SAR study, in which various functional groups on the small molecule of interest are altered or removed to assess which of these groups are essential for binding affinity. These non-essential groups can then be used as attachment sites for either an affinity tag, or a solid matrix. This component of affinity chromatography is one of the main limitations of the technique.
SAR studies are time-intensive, require a substantial amount of medicinal chemistry expertise, and often the small molecule cannot be chemically modified without impacting the bioaffinity. This approach, through total synthesis, allows access to novel and unique attachment sites which should not alter the binding affinity of the bioactive small molecule.

Alternatively, immobilisation of the ligand using photo-reactive groups is a faster way to capture the ligand either to an affinity tag or a solid matrix. This derivatisation technique captures the ligand in a site-non selective manner in multiple random orientations. However, if the ligand is directly attached to the solid support, no information can be accessed about the site of attachment or the degree of retained activity after immobilisation, which can make it difficult to assess any design problems if no binding proteins are found.

Once attached onto the solid support, the ligand becomes an affinity matrix to be used in affinity chromatography (Figure 1.7). The specially designed chemical probe, which can be likened to a molecular fishing rod, is garnished with a specific bait (small bioactive molecule) in order to capture a protein target or entire proteome subset.

![Figure 1.7 – The general structure of a typical affinity matrix.](image)

This process is known protein profiling, where through identification of the target biomolecule, information about the small molecule’s mode of action can be inferred. The method by which the ligand binds to the target biomolecule is either through non-covalent interactions such as affinity-based protein profiling (AfBPP) or covalent bonding using activity-based protein profiling (AcBPP) or photoaffinity labelling (PAL).
Non-covalent interactions

Affinity-based protein profiling

Many bioactive small molecules bind to cellular protein targets reversibly and form a transient small molecule-protein complex ($K_d = 10^{-3} \text{ M to } 10^{-7} \text{ M}$), which can be purified using affinity chromatography using an affinity-based protein profiling (AfBPP) probe.

A well-known early example of using small molecule bioaffinity ligands in affinity chromatography was reported by the Schreiber group.\textsuperscript{34}

![Rapamycin and FK506](R = CO(CH$_2$)$_2$C$_6$H$_4$NH-Affigel-10)

**Figure 1.8** – The structures of the rapamycin 1 and FK506 2 affinity matrices.\textsuperscript{34}

Building on previous work,\textsuperscript{35} the aim of their investigations was the identification of the binding proteins (immunophillins) found in T lymphocytes, which are responsible for the biological activity of the immunosuppressant rapamycin 1 and the structurally similar macrolide FK506 2 (Figure 1.8). Both molecules were attached to affinity matrices and incubated with Jurkat T cells. The predominant rapamycin binding protein was revealed to be the immunophillin FK506 binding protein (FKBP); this discovery proved that this immunophillin has a prominent role in mediating the biological activity of the drug, and mutual inhibition of the two related macrolides; rapamycin 1 and FK506 2.

The Schreiber group have also managed to identify myriocin binding proteins by using a myriocin-based affinity matrix 3 (Figure 1.9).\textsuperscript{36}
The natural product myriocin potently induces apoptosis of a murine cytotoxic T lymphocyte cell line (CTLL-2) and inhibits serine palmitoyltransferase (SPT) activity in ribosomal extracts from the CTLL-2 cell line which is thought to induce sphingolipid biosynthesis. A myriocin derivative was bound to maleimide-functionalised agarose, and two proteins were captured; long chain base biosynthesis protein 1 and 2 (LCB1 and LCB2, mammalian homologues of SPT1 and 2) which are genetically linked to sphingolipid biosynthesis and thus directly responsible for SPT activity.

In a phenotypic screen for mitotic arrest, Dückert et al. identified an indoloquinolizine, centrocountin 1, which caused the formation of mitotic spindles in BSC 1 cells. After SAR studies, an AfBPP probe 4 was synthesised and followed by incubation, pull-down and mass spectrometric identification of bound proteins found (Figure 1.10).

The results showed centrocountin 1 binds to the nucleolar and centrosomal protein nucleophosmin (NPM) and the U2 small nuclear ribonuclear protein (snRNP), which leads to impairment of centrosome and spindle integrity, chromosome congression defects and cell cycle arrest at the M stage of apoptosis. Target identification and mechanism of action of: tubulexin A (CSE1L, CAS, exportin-1 and tubulin), piperlongumine (GSTP1), and thalidomide (CRBN, DDB1), amongst many others have also been carried out using AfBPP.
Affinity Chromatography

**Covalent interactions**

**Activity based protein profiling (AcBPP)**

Often the transient binding of small molecule-protein complexes are much weaker ($K_d > 10^{-3}$ M), and unable to survive the *in vitro* affinity purification process needed for target identification. Activity-based protein profiling (AcBPP), established by the groups of Bogyo, and Cravatt, has become a useful tool in the biochemist’s toolbox, where active site-directed chemical probes are used for enzyme and inhibitor discovery. Small bioactive molecules selective for a particular reaction mechanism bind covalently to the active sites of enzymes or proteins. These AcBPP probes are often prepared with a tag for the purpose of enrichment (biotin) or visualisation (fluorescent dye). After the covalent binding to the target biomolecule, the probes allow for the straightforward analysis and identification by gel electrophoresis and/or fluorescent detection. AcBPP provides quick, sensitive and selective identification of enzyme activity and inhibitors in the proteome, where proteomic methods can only be used to determine the amount of a given protein/ enzyme in a cell at a given time, though not the activity.

![Figure 1.11](image_url)

- **Figure 1.11** – a) A typical activity-based protein profiling (ABPP) probe, and b) some examples of AcBPP reactive moieties and their target enzymes.

The design of a typical AcBPP probe is shown above (Figure 1.11, a). In its most basic form the AcBPP probe is comprised of three component parts: (i) the marker is used for the enrichment or visualisation of the target proteins, this can be biotin, for use in (S)Av affinity experiments, or a fluorescent dye; (ii) the spacer arm, which can be a
variety of different moieties, but most common is a hydrophilic PEG group, (discussed in detail in section 1.4.2); (iii) the reactive site (Figure 1.11, b), with a functional group which has been designed specifically for binding with the biomolecule of interest.47

In humans, several of the largest protein families (e.g. kinases, oxidoreductases, and hydrolases) are enzymes that are related by a common mechanism and/or structure.48 The reactive groups chosen for the AcBPP are based on their specificity for certain families of enzymes, examples of reactive moieties are shown in Figure 1.11 and AcBPP probes in Figure 1.12.

![AcBPP probes diagram](image)

**Figure 1.12** – AcBPP probes; a) fluorophosphonate-biotin (FP-Biotin) probe 10 used for isolation of serine hydrolases, b) DCG-04 activity probe 11 based on an irreversible inhibitor for cysteine proteases, and c) Acyloxymethyl ketone based probes 12-14 for cysteine proteases.46, 49, 50

Utilisation of the irreversible binding between fluorophosphonates (FP) and serine hydrolases permitted Liu et al. to synthesise a FP-based AcBPP probe 10 attached to a biotin reporter molecule (Figure 1.12, a).46 The enzyme must be in a catalytically active state in order to bind to FP, so they showed that FP-biotin can; (i) bind with several serine hydrolases in crude cell and tissue samples, (ii) be used to detect
subnanomolar concentrations of serine hydrolases, and (iii) record changes in both the functional state and expression level of the enzymes.

Greenbaum *et al.* constructed an AcBPP probe 11 based on an irreversible inhibitor E-64, an epoxide-containing natural product, which is broadly reactive towards the papain family of cysteine proteases (Figure 1.12, b). The epoxide inhibitor was coupled to biotin through a lysine residue side chain, and using affinity pull-down experiments, was able to profile cysteine protease activities in cell and tissue crude extracts. Finally, a recent study by Eitelhuber *et al.* designed AcBPP probes 12-14 for the detection of active MALT1 paracaspase in immune cells and lymphomas using an acyloxymethyl-based reactive group (Figure 1.12, c).

**Photoaffinity labelling**

When investigating the target protein of small bioactive molecules, it is hoped to identify the protein whose inhibition or activation leads to the biological response, while at the same time explore all proteins which bind to the compound, to investigate possible off-target effects of the drug. However, one of the shortcomings of affinity probes and biotinylated probes is the difficulty in isolating less abundant or low affinity proteins. Abundance problems can sometimes be remedied by using more protein; though isolation of low affinity proteins can be more difficult.

One way of resolving this problem is by using photoaffinity labelling (PAL), a technique developed over 40 years ago, but only now however being used to its full potential due to the development of new high-resolution separation and detection techniques and more efficient photophores. In PAL a photoreactive functional group is attached to the affinity matrix either through the linker or to the ligand (Figure 1.13, a). In this technique the PAL-AfBPP probe can be designed to bind specifically and reversibly to the biomolecule and following irradiation, generate a reactive intermediate which covalently cross-links to the target biomolecule. The new bond created by photoaffinity labelling (PAL) could give insight into the location and architecture of the binding site and potentially the interactome at the time of binding. The three most commonly employed photophores used in target identification are aryl azides, diazirines and benzophenones (Figure 1.13, b).
Affinity Chromatography

Figure 1.13 – a) Design of a photoaffinity probe, with possible sites of PAL attachment, b) commonly utilised photophores in photoaffinity experiments: (i) aryl azides are excited to singlet nitrenes ($X = H, F$); (ii) diazirines are irradiated to create carbenes; and (iii) benzophenones are irradiated to form radicals.

Aryl azides 15 are used in PAL due to their small size and the ease of introduction of the azido moiety. For PAL experiments using aryl azides, the course of the labelling reaction depends on the substituents on the aryl ring and the binding site architecture. A study from Bregant et al. synthesised a biotinylated AfBPP probe 18 targeting matrix metalloproteases (MMPs), using an MMP inhibitor as the ligand (Figure 1.14, a) to study the difference between affinity- and photoaffinity MMP enrichment from a complex proteome. It was demonstrated that after isolation on magnetic (S)Av beads, the affinity based labelling was superior to the photoaffinity probe, in terms of quantity of captured MMPs, due to poor crosslinking efficiency. The short excitation wavelength (<280 nm) required to activate the aryl azide is damaging to biological systems, and they are generally known to have poor crosslinking efficiency, thus aryl azides now are rarely used in PAL.

In comparison to aryl azides, diazirines 16 are activated at a much more biologically compatible wavelength (350 nm), whilst still being small in size. The carbene that is formed after irradiation is very reactive and crosslinks quickly and efficiently. Suzuki et al. describe the synthesis of photoactivatable and photocleavable beads which can be used to capture bioactive small molecules in a manner which is neither chemo- nor regio-selective upon irradiation (Figure 1.14, b). The PAL probe 19 used a two wavelength photoreaction system, where small molecules are immobilised onto
the probe by a primary UV irradiation to activate an aryl diazirine moiety, which is used in a pull-down experiment. After incubation the immobilised small molecule is released as a coumarin (fluorescent) conjugate after a secondary irradiation (302 nm). The released coumarin-conjugated small molecules are then quantified and identified by LC-MS. Development of a library of probes generated in this manner was tested successfully by the affinity purification of proteins that bind to the cyclic peptide cyclosporin A

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**Figure 1.14** – Photoaffinity probes: a) biotin bound aryl azide based probe 18 for MMP enrichment, b) immobilised cleavable diazirine probe 19, c) biotin bound benzophenone based probe 20 used for hCAII purification.57, 59, 60

Benzophenones 17, unlike azido and diazirinyl moieties, are quite large, though they are activated at a biologically compatible longer wavelength (350-365 nm) to generate a diradical.58 Sakurai *et al.* designed a PAL-AfBPP probe 20 using a benzenesulfonamide ligand, a potent inhibitor of human carbonic anhydrase II (hCAII), derivatised on the linker by a photoactivatable benzophenone moiety, and coupled to the reporter biotin (Figure 1.14, c). This probe was used successfully in the purification of hCAII from cell lysates.60
Although the classical affinity purification approach using solid support-immobilised or biotin-bound probes remains significant (Figure 1.15, a-c), there are underlying issues, such as; difficulty in derivitisation of ligands, non-specific interactions with the solid support, and problems with probe design leading to sterically bulky probes plagued with low cell permeability. This has restricted the classical approach to experiments in cell lysates exclusively, the disadvantage being that the cellular landscape is disrupted and therefore the targets and activity cannot be fully characterised.

**Figure 1.15** – Affinity purification probes: a) compounds immobilised directly onto the solid support; b) compound is non-covalently immobilised onto the solid support using biotin-(S)Av interaction; c) compound is attached to a biotinylated photoreactive probe; d) compound is modified with a bioorthogonal tag (alkyne) for *in situ* labelling (biotin/fluorophore) with negligible perturbation; e) a trifunctional probe, with a PAL group to induce covalent crosslinking.

These problems can be solved through the application of bioorthogonal chemistry, such as the copper(I) catalysed Huisgen alkyne azide cycloaddition (CuAAC) reaction or the Staudinger ligation, allowing tandem labelling strategies in which biotin or a fluorophore is attached in a subsequent step (Figure 1.15, d). Bioorthogonal reactions show high selectivity, low background binding with biomolecules and can
be conducted under physiological conditions (ambient temperature and pressure, neutral pH, aqueous conditions).

By combining approaches, proteins are bound in vivo using cell permeable bioactive molecules which have been functionalised for bioorthogonal reaction (Figure 1.15, d-e). Following cell disruption, the labelled biomolecule is bound to the tag and analysed. CuAAC functionalised small molecule probes have been significant in the discovery of on and off-target profiling of many drugs and bioactive natural products.\textsuperscript{47, 65-67}

Alkynyl sterol probes based on cholesterol 21 (Figure 1.16, a) were used to efficiently tag Sonic Hedgehog protein (Shh) cholesterylation and allow visualisation and analysis using bioorthogonal ligation (CuAAC) to reporters. Shh overexpression is seen in many types of cancer and is known to be a driver of carcinogenesis. The alkynyl probe 22 was used in investigation into the role of lipidation on the activity of Shh.\textsuperscript{68}

\textbf{Figure 1.16} – Examples of bioorthogonal tags for affinity (a), and photoaffinity (b,c) binding experiments.
Zerumbone 23 is a bioactive phytochemical with diverse biological activities (anti-inflammatory, anti-cancer), the cellular targets of which up until recently were unknown. The cell-permeable small molecule was functionalised with an alkynyl tag to give probe 24 (Figure 1.16, b), and used to profile cellular targets in live cancer cells. The alkynyl tag was placed distal to the key reactive centres of the phytochemical. Many of these target proteins identified play key roles in vital cellular processes such as apoptosis and cell survival, and it is hypothesised that the compound exerts its biological effect through modulation of a variety of these targets in an interactome co-dependent and context-dependent way.

In the work by Shi et al., Dasatinib 25 (Figure 1.16, c), a reversible kinase inhibitor, was used as the ligand of an AfBPP probe for proteome-wide profiling of potential cellular targets. As with most protein kinase inhibitors, Dasatinib has been shown to inhibit several cellular targets to effect a biological response and thus the potential for off-target effects is much greater. Using an AfBPP probe 26 with a PAL diazirine moiety, Shi et al. were able to identify many more (> 50) putative protein targets of the drug. Further investigations by the Yao lab saw the development of a smaller alkynyl diazirine linker 27 (Figure 1.16, c), which was used to derivatise a library of known kinase inhibitors to probe the cellular proteome.
1.5 Affinity independent elution

Elucidation and identification of the bound protein is an important step in affinity- and activity-based protein profiling. A common component of an affinity probe is a biotin moiety, which facilitates the capture and/or immobilisation of the affinity target by utilising the high affinity (S)Av/biotin interaction. However, quantative protein recovery by classical elution requires harsh and denaturing conditions in order to disrupt this interaction which can lead to contamination from non-specific materials [(S)Av and endogenously biotinylated proteins], and the loss of structural information and activity from the protein partner.\(^2\)

Milder elution methods which do not rely on competitive ligand displacement, so-called affinity independent elution protocols are desirable. Cleavable linker strategies allow selective release of target proteins under biologically compatible conditions, establishing a cleaner protein purification with retention of biological activity.

1.5.1 Enzymatic cleavage

Santala et al.\(^72\) describe a novel elution method for the efficient recovery of antibody-displaying phages that have been captured with a biotinylated antigen on streptavidin-coated paramagnetic particles. When considering the isolation of the high affinity binders, the standard elution method employed had a very low recovery.\(^73\) Consequently they used a nuclease-cleavable DNA linker unit situated between the (S)Av and the paramagnetic bead surface (CELLection™ Dynabeads®, 28), with which, after treatment with DNase, they achieved a high recovery (60-90% increase in recovery) and low non-specific background interactions (Figure 1.17, a).
Figure 1.17 – Enzymatically cleaved linkers: a) The commercially available CELLection™ Dynabeads® 28, cleaved by DNAase 1, b) a biotin-DNA-N₃ probe 29, cleaved by restriction endonuclease PvuII (cleavage site in red), c) Trypsin cleavable arginine containing linker 30 (cleavage point in red), d) TEV protease cleavable linker 31, (cleavable site in red). 72, 74-76

An alternative example of using DNA as a cleavage point was reported by Zheng et al., 76 a biotinylated cleavable DNA oligo was functionalised with an azido moiety 29 and used for the enrichment of alkyne-tagged glycoproteins from mammalian cell lysates (Figure 1.17, b).

A protease (trypsin) cleavable linker 30 containing a multifunctional capture reagent was used for the affinity enrichment of myristoylated proteins (Figure 1.17, c). 74 This allowed for the identification of the myristoylated proteome and the quantitative analysis of dynamic changes in protein lipidation during vertebrate development. Speers et al. 75 developed a cleavable linker 31 with an internal peptide sequence that is recognised by tobacco etch virus (TEV) protease, and cleaves between glutamine and glycine residues in the peptide central motif (Figure 1.17, d).
1.5.2 Photolytic cleavage

Linkers which can be photolytically cleaved have one clear advantage over those which cannot – in that they can be cleaved and release the biomolecule without using any other exogenous reagents. Several properties are required for an efficient photocleavable linker:

- Reactivity – the photocleavable group should react selectively and efficiently with a variety of target molecules under mild conditions.
- Complexation – if the linker is conjugated to biotin, it should still retain (S)Av binding ability.
- Photocleavage – the intensity and duration of light irradiation for complete cleavage should be minimal.
- Substrate release – the photolytic cleavage should result in the cleavage of the target of the affinity study in an unaltered form.

**o-Nitrobenzyl derivatives**

o-Nitrobenzyl type derivatives are the most popular photolabile group, which is due to the high stability which they exhibit under a variety of different conditions (e.g. pH, Lewis acid/base), they are cleaved selectively and efficiently at 365 nm (LED/mercury lamp). However photolysis generates nitrosobenzene which can induce unwanted side reactions. The cleavage success is dependent on the easy 1,5-transfer of a hydrogen from carbon to oxygen, a Norrish type II process driven by the greater O-H bond strength versus C-H bond strength.

Olejnik *et al.* synthesized a photocleavable biotin-NHS ester 32 (PCB-NHS) reagent for labelling biomolecules, using an internal 1-(2-nitrophenyl)-ethyl moiety (Figure 1.18, a; cleavage site at arrow head). To demonstrate the PCB’s utility, it was coupled to model substrate [leucine]enkephalin using the NHS amide bond strategy (Figure 1.18, b). The PCB-Leu-Enk conjugate was shown to retain affinity for (S)Av, and after irradiation the substrate was released unaltered in a rapid and efficient manner.
Affinity Chromatography

Figure 1.18 – Photocleavable biotin derivative synthesised by Olejnik et al. a) NHS activated cleavable biotin 32, b) the pentapeptide [Leu]Enk was coupled using the PCB-NHS 33.

A photocleavable linker 34 by Koopmans et al.,\textsuperscript{80} based on work from the groups of Rothschild\textsuperscript{79, 81} and Tirrell,\textsuperscript{82} was designed incorporating a photosensitive 1-(2-nitrophenyl)-ethyl moiety with orthogonal reactive functionalities: an amine for linking to biotin, and an azide for CuAAC reaction with alkynyl ligands of interest (Figure 1.19, a).

Figure 1.19 – a) Structure of photocleavable tag 34 designed to cleave at $\lambda = 366$ nm, and b) CALB bound linker 35.\textsuperscript{80}

The linker was tested using a model system incorporating the well characterised family of fluorophosphonate probes to illustrate serine hydrolase activity.\textsuperscript{44, 46} A fluorophosphonate alkyne probe was synthesised, covalently bound to CALB (\textit{Candida antarctica} lipase B) and clicked onto the photocleavable tag 34 to give conjugate 35 (Figure 1.19, b). Two different cleavage mechanisms were used: i) heat and SDS to disrupt the biotin/(S)Av interaction, and ii) irradiation (366 nm, 0 °C). The irradiation cleavage mechanism provided a much cleaner recovery, as the SDS elution sample (40 kDa) was contaminated by a low molecular weight (14 kDa) impurity from (S)Av.
**Novel photocleavable linker**

Another example of a biologically compatible photocleavable linker has been demonstrated by Yonezawa *et al.* A novel and effective photocleavable linker based on a core α-thioacetophenone structure was designed. The linker 36 was synthesised with orthogonally protected amino moieties (Figure 1.20, a), and undergoes a Norrish type II cleavage reaction after irradiation at a more biologically compatible wavelength (365 nm).

![Figure 1.20](image)

*Figure 1.20* – a) Design of the α-thioacetophenone linker 36, b) application of the linker.

The cleavage efficiency was first tested using the TAMRA bound linker 37 (Figure 1.20, b), and monitored by fluorescence spectroscopy. After irradiation the cleavage yield reached a maximum of 45% after 1 h. The cleavable linker was applied to the fluorophosphonate (FP) model system, where in place of the fluorophore, a FP moiety was attached via an alkyl chain 38. FPs are known to bind to serine hydrolases, and so the affinity linker was used to purify trypsin with a recovered yield of 61%. This shows that the novel α-thioacetophenone can be added to the repertoire of light cleavable moieties for application in chemical biology.
1.5.3 Chemical cleavage

Disulfide bond reduction cleavage

Disulfide bonds are stable in a variety of different biological environments, and are cleaved selectively using glutathione or DTT. This reductive cleavage requires some caution, as some protein disulfide bonds may be affected in the reduction step and resulting in a loss of quaternary protein structure.

Figure 1.21 – Disulfide-based cleavable linkers, a) with aryl diazirine for PAL, b) with cyclooctyne for strain promoted [3+2] cycloaddition to azides.\textsuperscript{84, 85}

Kanoh \textit{et al.}\textsuperscript{84} used a solid-supported disulfide cleavable (and photoactivatable) linker 39 to immobilise (in a non-selective manner) small molecules onto an affinity matrix (Figure 1.21, a). The cleavable site was introduced to verify the presence of the small molecule on the solid support, and also to isolate small molecule protein targets for purification. This affinity matrix was then tested successfully to find the binding proteins of FK506 and cyclosporin A. Nessen \textit{et al.}\textsuperscript{85} described the use of a disulfide cleavable solid supported linker 40 used for the selective enrichment of azide-containing peptides from complex peptide mixtures (Figure 1.21, b).

Azobenzene linkers

First established by Verhelst\textsuperscript{86}, and further developed by Hang,\textsuperscript{87-89} Hulme,\textsuperscript{90} Bogyo,\textsuperscript{86} and Wagner\textsuperscript{6} groups, the azobenzene unit is chemically cleaved to give two aniline cleavage products under mild conditions using the biologically compatible reagent sodium dithionite.
Figure 1.22 – Comparison of three azobenzene strategies.\textsuperscript{6,86,90}

The strategy utilised by the Bogyo group 41 enabled the purification of cathepsin peptides from rat liver homogenate (Figure 1.22, a).\textsuperscript{86} The Hulme group linker 42 incorporated an azide functionality which can undergo CuAAC with a series of alkynyl-functionalised compounds of biological interest (Figure 1.22, b).\textsuperscript{90} The affinity probe 43 developed by the Wagner group was used to obtain the bacterial type II topoisomerase, DNA gyrase, by coupling the inhibitor aminocoumarin novobiocin as the ligand (Figure 1.22, c).\textsuperscript{6}
1.6 Results

1.6.1 Linker strategy

Previous work carried out in the Hulme group with cleavable linker 42, had tested its robustness by coupling to an Affi-gel 10 (Bio-Rad) solid support 44 (Figure 1.23) and subsequent CuAAC reaction to a propargyl amine derivative of biotin 45, to create an affinity matrix 43 used successfully in an avidin enrichment experiment.\(^9\)

Due to the success of the linker in these initial studies, the next step was to utilise it further in protein target identification of CuAAC-functionalised natural product analogues (Chapter 2 and 3). Towards this end, a selection of linkers which incorporate PAL were synthesised to generate a toolbox of AfBPP probes (through CuAAC coupling) for use in affinity chromatography.
1.6.2 Linker synthesis

Affinity independent probes

The novel azobenzene linker 42 was prepared on gram scale as the HCl salt using the concise synthesis published in Landi et al. (Figure 1.24).90

![Diagram of linker synthesis]

**Figure 1.24** – Linker synthesis. Reagents and conditions: (a) i. NaNO₂, HCl, 0 °C, 20 min; ii. PhOH, NaOH, K₂CO₃, 0 °C to rt, 3 h, 85%; (b) BocNHCH₂CH₂NH₂, EDC, HOBt, DMF, rt, 18 h, 62%; (c) 1,3-dibromopropane, K₂CO₃, CH₃CN, reflux, 5 h, 65%; (d) NaN₃, DMF, rt, 18 h, 89%; (e) MeOH, CH₃COCl, Et₂O, 0 °C to rt, 1 h, 92%.

The azobenzene core 48 was accessed from the diazotisation of 4-aminobenzoic acid 47 and reaction with phenol.91 Subsequent amide bond formation with N-Boc-ethylenediamine and alkylation of the phenol with 1,3-dibromopropane, gave the alkyl bromide 49 in good yield. The azido moiety was introduced by nucleophilic displacement of alkyl bromide with sodium azide to give compound 50, the reaction was monitored using ESI-MS as the starting material and product had an identical R_f. Deprotection of the Boc group gave the final cleavable linker 42 in 5 steps and 28% overall yield (Figure 1.24).

As an alternative to immobilisation onto a solid support, the linker 39 was coupled through an amide bond to biotin 48 to make the affinity independent probe 50, to exploit the biotin-(S)Av interaction and take advantage of the variety of (S)Av immobilised solid supports available (Figure 1.25).
Two routes were pursued for the coupling; and the amide bond formation proceeded in comparable yields for coupling to both D-biotin 51 and biotin-NHS 52. However, HBTU-mediated coupling\(^{92}\) of acid 51 (Figure 1.25) gave quicker reaction times, using the relatively inexpensive starting material D-biotin 51.

With the aim of investigating low affinity or low abundance target proteins, the biotinylated cleavable linker 50 was altered to enable the coupling of the photoactivatable moieties: alkyl diazirine and benzophenone. Modification of the linker synthesis to include a photoreactive moiety, by change of the phenol alkylation reagent (Figure 1.26) resulted in poor yields and a convoluted synthesis scheme.
A more concise alternative, the azide 53 was modified by Staudinger reaction to give the amine 54 (Figure 1.27) which might be used to attach a PAL via amide bond formation.\textsuperscript{93,94} The reduction of azide 53 was found to be very capricious, and many attempts at modifying the reaction conditions were carried out.

![Chemical structure](image)

**Figure 1.27** – Reduction of biotin-linker-N\textsubscript{3} by Staudinger reaction. Reagents and conditions: (a) PPh\textsubscript{3}, 10:1 THF/H\textsubscript{2}O, rt, 18 h, 25-67%; or (b) TCEP, Et\textsubscript{3}N/MeOH/H\textsubscript{2}O, rt, 5 h, 60%.

Firstly the method shown in Figure 1.27 was carried out with an acid-base work-up, though due to the high solubility of biotin-linked compounds in aqueous solutions and very low solubility in organic solvents, the yield of product 54 was low. Next, a solid-supported PPh\textsubscript{3} was used,\textsuperscript{95} but again the yield was poor, this was due to the retention of the iminophosphorane-linker on the resin (orange resin beads). Using LiAlH\textsubscript{4} as the azide reducing agent resulted in a complex mixture of products, which was comprised of starting material and cleaved linker but no product. Finally by repeating the original method, with an alternative work-up of directly dry-loading silica with the reaction mixture and chromatography under basic conditions (5:10:85, Et\textsubscript{3}N/NH\textsubscript{3}/MeOH:DCM) the linker amine 54 was isolated in moderate yield. A further reduction method was attempted using tris(2-carboxyethyl)phosphine (TCEP), a water-soluble reducing agent frequently used in biological applications (reductive cleavage of disulfide bonds in proteins), which can also be used to reduce azides.\textsuperscript{96} The reaction proceeded well, in part due to the solubility of the biotin linker azide 53 in the Et\textsubscript{3}N/MeOH/H\textsubscript{2}O mixture.
The amino group provided an attachment point for a branched compound (acid moiety for amide coupling, azide moiety for CuAAC coupling, and a functional group for photoactivatable group attachment). Amino acids are a natural source of branching and easily functionalised, thus an orthogonally protected lysine 55 (Fmoc-Lys(Boc)-OH) was chosen as the basis of the branching compound (Figure 1.28).

Figure 1.28 – Preparation of azido-lysine derivatives. Reagents and conditions: (a) EDC, HOBt, MeOH, rt, 18 h, 85%; (b) i. TFA, DCM, rt, 3 h; ii. 57, K2CO3, CuSO4•5H2O, MeOH, rt, 18 h, 64%; (c) DBU, DMF, rt, 3 h, 95%; (d) piperidine, DMF, rt, 3 h, 90%.

Fmoc-protected lysine 55 was esterified using EDC/HOBt and methanol to the methyl ester 56, acidic esterification conditions were avoided due to the acid-sensitive Boc protecting group on the lysine side chain. After Boc deprotection, the primary amine was converted to an azide using imidazole-1-sulfonyl azide hydrochloride 57 as a azide transfer reagent97 to give Fmoc-Lys(N3)-OMe 58, which was further deprotected by the removal of Fmoc by DBU to give azido amine 59. In addition, H-Lys(Boc)-OMe 60 was isolated by piperidine deprotection of methyl ester 56 as an alternative for photoreactive moiety coupling.

The photoreactive groups for inclusion in the affinity ‘toolbox’ were chosen dependent on their ease of synthesis and compatibility with CuAAC coupling. Consequently, the two compounds chosen were 4-benzoyl benzoic acid 61, a commercially available benzophenone moiety, and 3-(3-methyl-3H-diaziren-3-yl)propanoic acid 63, synthesised in 1 step from levulinic acid 62 (Figure 1.29).98
4-Benzoyl benzoic acid 61 was coupled to lysine-derived amino azide 59 to give benzophenone azide 65 in an excellent yield with standard EDC/HOBt coupling conditions (Figure 1.30), care was taken not to expose the benzophenone compounds to light. Following basic hydrolysis of the methyl ester (Figure 1.29, d), the carboxylic acid 66 was released, in a good yield (5 steps from Fmoc-Lys(Boc)-OH, 44% overall yield).

An alternative synthesis was also investigated (Figure 1.30, b-c), by first coupling the 4-benzoyl benzoic acid 61 to H-Lys(Boc)-OMe 60 (derived in one step from Fmoc-Lys(Boc)-OMe, Figure 1.28, d) to give the Boc-protected amine 64, then using the previously described conditions for the diazotransfer reaction (Figure 1.28, b) to give benzophenone azide 65. Basic hydrolysis of the methyl ester gave the carboxylic...
acid 66 with a comparable overall yield (5 steps from Fmoc-Lys(Boc)-OH, 40% overall yield).

The same approaches were used in the synthesis of diazirine azido acid 69 (Figure 1.31), though the overall yields are much lower (coupling to: 60, 6 steps from Fmoc-Lys(Boc)-OH, 12% overall yield; 59, 6 steps from Fmoc-Lys(Boc)-OH, 14% overall yield) indicating that the diazirine moiety is much more readily degraded. This comparative instability is reinforced by ESI-MS data (loss of $m/z = 26$), which reflects the loss of the diazirine.

![Figure 1.31 – Synthesis of diazirine azido-lysine derivatives. Reagents and conditions: (a) 59, EDC, HOBt, NEt$_3$, DMF, rt, 18 h, 65%; (b) 60, EDC, HOBt, NEt$_3$, DMF, rt, 18 h, 84%; (c) i. TFA, DCM, rt, 3 h; ii. 57, K$_2$CO$_3$, CuSO$_4$•5H$_2$O, MeOH, rt, 18 h, 60%; (d) NaOH, 1:1 MeOH/H$_2$O, rt, 2 h, 60%.](image)

With the photoactivatable branched azides 66 and 69 in hand, the affinity independent biotinylated linkers 70 and 71 were synthesised (Figure 1.32) and added to the toolbox. The yields for the linkers 70 and 71 were lower than expected for a standard amide bond formation (26% and 30% respectively). This was partially due to solubility issues of the probes both during the reaction and purification of the final compounds.
Figure 1.32 – Synthesis of AfBPP-PAL tags 70 and 71. Reagents and conditions: (a) EDC, HOBt, NEt$_3$, DMF, rt, 48 h, 70 26% 71 30%.

**Affinity dependent probes**

A series of affinity-dependent probes without the chemically cleavable azobenzene linker were synthesised to evaluate the efficiency of the cleavable probes. Amino azide 74 is readily available from the hydrochloride salt 73, as reported in the literature.$^{101}$ D-biotin 51 was coupled directly to aminoazide 74 under microwave irradiation, to generate the biotin azide 75 in satisfactory yield (67%).

Figure 1.33 - Synthesis of 3-azido-1-propylamine 74 then subsequent coupling with D-biotin 51. Reagents and conditions: (a) NaN$_3$, H$_2$O, 80 °C, 18 h, 98%; (b) HOBt, DIC, DMF, 51, µwave, 60 °C, 20 min, 67%.

In order to evaluate the utility of the affinity-independent PAL linkers, the synthesis of affinity-dependent probes incorporating the same PAL groups was proposed. NHS-activated biotin 52, which reacted with ethylenediamine under alkaline conditions to give biotin amine 76 in good yield (Figure 1.34). Biotin amine 76 was amide coupled (EDC/HOBt) to the photoactivatable lysine derivatives: benzophenone 66 and
diazirine 67, to produce the biotinylated affinity dependent linkers 77 and 78 (Figure 1.34).

Figure 1.34 – Synthesis of biotin amine 76 and biotinylated photoaffinity linkers 77 and 78. Reagents and conditions: (a) ethylenediamine, DIPEA, DMF, rt, 18 h, 90%; (b) EDC, HOBT, DIPEA, DMF, rt, 48 h, 77 32% 78 35%.
1.7 Discussion

A series of biotinylated affinity linkers, functionalised with an azide for use in the CuAAC reaction were synthesised (53, 70, 71, 75, 77, and 78). Within this series, three biotin-anchored affinity-independent linkers with an azido moiety were synthesised using an azobenzene core unit which has precedent within the Hulme group (53, 70 and 71). In order to increase the chances of identifying low affinity, or low abundance, binding proteins of target molecules, this series includes diazirine and benzophenone modified photoaffinity linkers (70 and 71). These linkers can be used in affinity-independent elution protocols; as the azobenzene core unit can be cleaved under mild, non-denaturing conditions (elution with Na₂S₂O₄). Three further affinity linkers, which can be used in affinity-dependent elution protocols, have also been synthesised to compare the efficiency of the linkers previously made (75, 77, and 78). These affinity-dependent linkers again include diazirine and benzophenone modified photoaffinity linkers (77, and 78). Together these six linkers form a toolbox for use in pull-down studies.

There were several complications when trying to synthesise the affinity linker toolbox; the two major problems being solubility of the linkers and the light sensitivity of the photoaffinity linkers. Biotin is easily introduced, however biotinylated linkers are very hard to handle, requiring very polar solvents (DMF/DMSO/MeOH) in order to solubilise them. The biotinylated cleavable linkers were the most difficult to handle, however the linker is bright orange and has a very strong UV absorbance at 358 nm, and thus is easily identifiable by TLC and HPLC. Perhaps inclusion of biotin at the final stage of the affinity linker toolbox synthesis would improve the ease of synthesis and purification. Azobenzene has long been known and used as a dye because of its intense orange colour, but one of its most intriguing properties is the photoisomeriation to both cis and trans isomers with UV irradiation. The trans isomer is the most thermally stable of the two, and can be regenerated from the cis isomer by blue light (>400 nm). The presence of the cis isomer was seen towards the end of the linker synthesis by ¹H NMR, however after blue light irradiation (by LED) the product was fully restored to the trans isomer (as confirmed by NMR).
The azobenzene core unit 42 was synthesised in 5 steps and 28% overall yield, benzophenone-Lys(N\textsubscript{3})-OH 66 was synthesised in 5 steps and 40% overall yield, and diazirine-Lys(N\textsubscript{3})-OH 69 was synthesised in 6 steps and 14% overall yield. The overall yields of the affinity linker toolbox using the substrates above are tabulated below.

<table>
<thead>
<tr>
<th>Affinity linker</th>
<th>Steps from core unit</th>
<th>Overall Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biotin-linker-azide 53</td>
<td>1</td>
<td>86%</td>
</tr>
<tr>
<td>Biotin-linker-diazirine-azide 70</td>
<td>3</td>
<td>From Diazirine-Lys(N\textsubscript{3})-OH; 34%</td>
</tr>
<tr>
<td>Biotin-linker-benzophenone-azide 71</td>
<td>3</td>
<td>From Benzophenone-Lys(N\textsubscript{3})-OH; 31%</td>
</tr>
<tr>
<td>Biotin-azide 75</td>
<td>n/a</td>
<td>65%</td>
</tr>
<tr>
<td>Biotin-diazirine-azide 77</td>
<td>n/a</td>
<td>From Diazirine-Lys(N\textsubscript{3})-OH; 29%</td>
</tr>
<tr>
<td>Biotin-benzophenone-azide 78</td>
<td>n/a</td>
<td>From Benzophenone-Lys(N\textsubscript{3})-OH; 32%</td>
</tr>
</tbody>
</table>

*Table 1.2 – Summary of affinity linker toolbox synthesis*

With respect to other affinity linkers in the field, the toolbox synthesised is in part unique, due to it being cleavable and photoreactive (77 and 78). The complete toolbox of affinity linkers which have been synthesised, varying in length, cleavability and photoreactive group utilised, is more varied than attempted by other research groups, with the hope of finding low and high affinity binding proteins to ligands of interest.
Chapter 2  Anisomycin

2.1 Introduction

2.1.1 Natural products in drug discovery

Natural products have been used as drugs for their medicinal, recreational and lethal properties for millennia, and until recently all drugs were based upon natural products. A review by Newman and Cragg extensively details NPs as sources for drugs, where in the area of cancer from the 1940s to 2010, 48.6% of the drugs approved were natural products, or directly derived therefrom. This review advocates expanding the exploration of nature as a source of novel active agents that may serve as scaffolds and leads for elaboration into urgently needed efficacious drugs.

2.1.2 Drug repurposing

Diseases such as cancer are rapidly growing resistant to existing clinical treatments, thus the need to find ‘new’ drugs is a pressing concern. In drug discovery; target validation to clinical application of a single drug can take an average of 15 years, and US$1.8 billion (data from 68 FDA approved drugs). Despite increasing capital invested in research spending by the NIH, the number of new chemical entities (NCEs) approved by the FDA in the past 5 years have seen a decrease (50% fewer NCEs than the previous 5 years). Thus, approaches which cut down the drug discovery time-frame, increase success rates and decrease spending are required. Drug repurposing (or repositioning) is a strategy which utilises the current pharmacopedia to screen for new clinical uses. These clinically ‘old’ or abandoned drugs have detailed information regarding potential toxicity, pharmacokinetics and pharmacodynamics profiles from previous R&D endeavours, thus enabling faster testing of new hypotheses and rapid translation into phase II/III clinical trials. Additionally, the pharmacologist, Nobel laureate and fellow Scot, Professor Sir James
Black famously once said, "The most fruitful basis for the discovery of a new drug is to start with an old drug."112

<table>
<thead>
<tr>
<th>Drug</th>
<th>Original Use (mechanism)</th>
<th>Repurposed (mechanism)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azidothymidine</td>
<td>Cancer (RT ↓)</td>
<td>HIV (HIV-1 RT ↓)</td>
</tr>
<tr>
<td>(AZT)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalidomide</td>
<td>Antiemetic in pregnancy (TNF-α ↓)</td>
<td>Leprosy (TNF-α ↓)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AIDS (TNF-α ↓)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cancer (NF-κB ↓, STAT3 ↓)</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>Pulmonary hypertension (PDE5 ↓)</td>
<td>Erectile dysfunction (PDE5 ↓)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>Analgesic, antipyretic (COX-1 ↓, COX-2 ↓)</td>
<td>Colorectal cancer (COX-2 ↓, NF-κB ↓, AP-1 ↓)</td>
</tr>
<tr>
<td>Statins</td>
<td>Myocardial infarction (HMG-CoA reductase ↓)</td>
<td>Prostate cancer, leukemia (NF-κB ↓, HMG-CoA reductase ↓)</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Immunosuppressant (mTOR ↓)</td>
<td>Colorectal cancer, leukemia, lymphoma (NF-κB ↓, IL-6 ↓, IKK ↓)</td>
</tr>
<tr>
<td>Nitroxoline</td>
<td>Antibiotic</td>
<td>Bladder, breast cancer (MetAP-2 ↓)</td>
</tr>
</tbody>
</table>

Table 2.1 – Brief overview of repurposed drugs.106, 111 Abbreviations: RT, reverse transcriptase; TNF-α, tumour necrosis factor alpha; NF-κB, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3; PDE5, cGMP-specific phosphodiesterase type 5; COX-1/2, cyclooxygenase-1/2; AP-1, activator protein 1; mTOR, mechanistic target of rapamycin; IL-6, interleukin 6; IKK, IκB kinase; MetAP-2, Methionine aminopeptidase 2; ↓, downregulation.

The paradigm “one gene, one disease, one drug” which was once the central dogma of drug discovery, has now been challenged with the concept of polypharmacology where drugs act upon multiple targets rather than one target.113 This theory leads to drug repurposing, as exemplified in Table 2.1 above, where one drug can have several biological targets and thus new drug-disease associations.
2.1.3 Anisomycin

Anisomycin (ANS, 79) can be considered in the area of drug repurposing due to its recently discovered anti-cancer activity by activation of MAPK/SAPK cellular pathways, which is markedly different to its original proposed use as an antibiotic and topical antifungal treatment.\textsuperscript{114} The pyrrolidine based natural product ANS was first reported by Sobin and Tanner in 1954.\textsuperscript{115} Initially isolated from cultures of \textit{Streptomyces roseochromogenes} and \textit{Streptomyces griseolus}, it was shown to possess selective and potent activity against pathogenic protozoa and certain strains of fungi, also inhibiting \textit{Entamoeba histolytic}, \textit{Trichomonas vaginalis}, \textit{Tritrichomonas foetus} and \textit{Candida albicans}.\textsuperscript{116}

\begin{figure}[h]
\centering
\includegraphics[width=0.2\textwidth]{anisomycin.png}
\caption{The natural product anisomycin, 79}
\end{figure}

ANS elicits several other interesting biological responses, such as anti-tumour activity with \textit{in vitro} cytotoxicity at nanomolar concentrations. ANS also inhibits protein synthesis both \textit{in vivo} and \textit{in vitro} by peptidyl transferase inhibition, binding to the 60S ribosomal subunit in eukaryotes.\textsuperscript{117-119} Perhaps the most interesting activity of anisomycin is the potent activation of the MAPK/SAPK cascades which it induces at levels below those required for protein synthesis inhibition (typically < 3 µM, Figure 2.2).\textsuperscript{120-123}

Mitogen activated protein kinase (MAPK) pathways form the backbone of signal transduction in the mammalian cell. The pathways are a collection of protein signalling cascades activated by a wide variety of extra cellular signals, including growth factors, cytokines and environmental stresses. A human MAPK interactome has recently been described by Bandyopadhyay \textit{et al.} detailing the discovery of more than 2000 protein interactions related to MAPK signalling.\textsuperscript{124}
MAPK pathways regulate many fundamental cellular functions, such as differentiation and proliferation, and have been implicated in the pathogenesis of cancer and autoimmune diseases due to their central role in signal transduction. This is why they are good targets for drug discovery. The MAPK family includes the extra-cellular signal regulated kinases (ERK1 and 2), and the stress activated kinases (SAPKs) including c-Jun N-terminal kinase (JNK/SAPK1) and p38 kinase (p38/SAPK2). Transcription factors such as c-Jun, ATF-2 and ELK-1 are activated by phosphorylation by activated SAPKs, and the downstream effects of this are thought to play a role in a range of pathological disorders such as Alzheimer’s disease and cancer. The downstream effects on the SAPK pathway induced by anisomycin have been well documented, although the cellular target of anisomycin and thus effector mode of activation has yet to be elucidated.
Figure 2.3 – Mitogen activated protein kinase cascades.
2.1.4 Previous biological work

To determine which structural modifications made to the anisomycin core retained SAPK phenotype activity, SAR analysis was performed using a series of ANS analogues to determine the levels of activation of the SAPK pathway.\textsuperscript{127} Assessment of the phosphorylation of c-Jun and MAPKAP-K2, was carried out by immunoblot assays with cellular stimulation at “sub-inhibitory” concentrations (30 µM, Table 2.2).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Analogue & R\textsuperscript{1} & R\textsuperscript{2} & R\textsuperscript{3} & R\textsuperscript{4} & c-Jun\textsuperscript{a} & MAPKAP-K2\textsuperscript{b} & JNK1/2\textsuperscript{c} \\
\hline
ANS\textsuperscript{d} & H & OAc & OH & OMe & +++ & +++ & +++ \\
\hline
EMR112 & H & OAc & H & OMe & +++ & +++ &  \\
\hline
EMR94 & H & OAc & Me & OMe & ++ & ++ &  \\
\hline
EMR59 & H & OCOEt & OH & OMe & +++ & +++ &  \\
\hline
EMR14 & H & OH & OH & OMe & + & + & + \\
\hline
EMR56 & Bn & OH & OMe & OMe & + & - &  \\
\hline
IAI140, 80 & OCH\textsubscript{2}C≡CH & OAc & H & OMe & ++ &  \\
\hline
IAI138 & H & OAc & H & OMe & ++ &  \\
\hline
IAI136, 81a & H & OAc & OH & OCH\textsubscript{2}C≡CH & ++ &  \\
\hline
IAI141 & Bn & OAc & OH & OMe & + &  \\
\hline
\end{tabular}
\caption{Overview of ANS analogues tested. Testing in murine RAW macrophages with antibodies which recognise: \textsuperscript{a} c-Jun phosphorylation at Ser63, and \textsuperscript{b} MAPKAP-K2 phosphorylated at Thr334. Testing in HEK293 cells with an antibody that recognises \textsuperscript{c} JNK1/2 phosphorylation of at Thr183. \textsuperscript{d} ANS tested was purchased from Sigma Aldrich.}
\end{table}

ANS analogues were tested in two separate campaigns,\textsuperscript{28,127} using RAW macrophage and HEK 293 cell lines. The initial RAW macrophage assays showed that ANS and some analogues activated phosphorylation of c-Jun and MAPKAP-K2 (Table 2.2, Figure 2.2).\textsuperscript{127} In subsequent HEK 293 cell assays, activation of the JNK/SAPK1 pathway was assessed using an antibody specific for phosphorylation of JNK1/2.
Anisomycin

In a further study by the Hulme group, a propargyl group was attached to both the pyrrolidine nitrogen of ANS and the phenolic oxygen of H-form ANS (80a and 81a respectively) in a view to synthesising biotinylated molecular probes. Both N-propargyl ANS 80a and the derived molecular probe biotin-C3-triazole-anisomycin compound 80b showed levels of activation equivalent to that of anisomycin itself, while derivation of the O-linked precursor 81a to give 81b led to a loss of phenotypic response (Figure 2.4).

Anisomycin analogues (Table 2.3) which had previously showed moderate to strong activation of the JNK/SAPK1 pathway in HEK 293 cells and RAW macrophages were supplied to Dr Howard Fearnhead (NUIG). ANS was identified in a screen of clinical compounds (Johns Hopkins Clinical Compound Library, JHCCL) as a drug which killed breast cancer cells. The analogues activities were tested by considering cell viability (mitochondrial activity) and JNK phosphorylation in triple negative breast cancer cell lines. Triple negative breast cancer (TNBC) is characterised by tumours that do not express the genes for the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2), and signifies an important clinical struggle as these cancers do not respond to endocrine therapy or other targeted agents. TNBC is characterized by distinct molecular, histological and clinical features including a particularly unfavourable prognosis despite increased sensitivity to standard cytotoxic chemotherapy regimens.
Anisomycin

Table 2.3 – Overview of ANS analogues tested at NUIG. Testing carried out in MDA-MB-468/231 TNBC cell lines; \(^a\) protein synthesis inhibition assessed by SUnSET assay, \(^b\) cell death assessed by colony formation, \(^c\) JNK phosphorylation evaluated using Thr183/Tyr185-JNK antibody, \(^d\) cell death assessed by mitochondrial activity. Compounds HOF25 and HOF17 are control compounds with structures related to anisomycin which have been previously shown to be inactive.

The work recently carried out by the Fearnhead group has shown that most of the anisomycin analogues decrease mitochondrial activity in the two triple negative cell lines used (MDA-MB-468 and MDA-MB-231), and did not appear to induce phosphorylation of JNK.\(^{131}\) Both anisomycin 79 and the propionate analogue 85 induced the phosphorylation of JNK, though analogue 85 did not induce cell death. Analogues 82, 83, and 84 induced cell death, though did not activate the JNK pathway. These results in the triple negative breast cancer cell lines were somewhat unexpected, based on earlier assays in HEK293 cell lines and RAW macrophages, and suggest that perhaps ANS-induced JNK phosphorylation is not the sole mechanism for ANS-induced cell death.\(^{131, 132}\) It is possible that the interactome of these triple negative breast cancer cell lines differs slightly from the interactome in the HEK 293 cells, leading to alternative cell-death mechanisms.\(^{133}\) Following these data, it was hoped that by using affinity chromatography, the proteins creating the phenotypic response could be elucidated.
2.2 Results

2.2.1 Anisomycin affinity probes

Click chemistry was the conjugation method of choice for coupling the ANS to the affinity linker toolbox by using the copper(I) catalysed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction. The reason for choosing this method lies within its many benefits, such as: high reliability, the ease with which azides and alkynes are introduced into organic molecules, the bioorthogonal properties of the reactive functional groups and the mild reaction conditions. This cycloaddition between a terminal or internal alkyne and an azide creates the 1,2,3-triazole as its 1,4-regioisomer selectively.

![Figure 2.5](image_url)  
Figure 2.5 – Copper catalysed Huisgen 1,3-dipolar cycloaddition reaction forming the 1,4-triazole regioselectively.

Tris-(benzyltriazolylmethyl) amine (TBTA) was used as stabilising ligand for the copper(I) species in CuAAC coupling, protecting it from oxidation [to Cu(II)] and disproportionation [to Cu(0) or Cu(II)] by enveloping the copper(I) centre to leave no free binding sites for potential destabilising reactions, while enhancing the catalytic activity. SAR analysis of ANS had previously shown that alkylation of the nitrogen on the pyrrolidine ring would retain biological activity. In this study a propargyl derivative of ANS was synthesised for use in the CuAAC reaction. The reaction was carried out in the absence of light so as to prevent polymerisation and photolysis of the reactant propargyl bromide.
Figure 2.6 – Propargylation of anisomycin. Reagents and conditions: (a) propargyl bromide, potassium carbonate, DMF, rt, 8 h, dark, 95%.

The synthesis of biotinylated ANS affinity probes were performed using the synthesised affinity linker toolbox (Chapter 1) and propargyl ANS 80a using CuAAC coupling (Figure 2.4). Two Cu(I) sources were used – copper(II) sulfate hexahydrate (reduced to Cu(I) species by sodium ascorbate), and tetrakis(acetonitrile)copper(I) tetrafluoroborate.138,139

The biotin azide 75 so-formed (Section 1.6.2), was coupled to propargyl ANS 80a under the TBTA-assisted CuSO₄/NaAsc CuAAC protocol to give the biotin-C₃-triazole anisomycin compound 89.140 The biotin-linker-azide 54 was then coupled to the propargyl ANS 80a under the same optimised TBTA-assisted CuSO₄/NaAsc CuAAC protocol to give the biotin linker anisomycin compound 92. Both of these reactions gave solubility issues in the H₂O/t-BuOH solvent mixture, and thus the yields were not satisfactory. So the alternative CuAAC strategy was employed using the Cu(I) species Cu(MeCN)₄BF₄, which is soluble in organic solvents, such as DMF and DCM.141 The reactions now proceeded with a much greater yield, and reduced reaction time (Table 2.4).

<table>
<thead>
<tr>
<th>Azide</th>
<th>Alkyne</th>
<th>Cu Source</th>
<th>Solvent</th>
<th>Time</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>80</td>
<td>CuSO₄</td>
<td>H₂O/t-BuOH</td>
<td>24 h</td>
<td>59%</td>
</tr>
<tr>
<td>54</td>
<td>80</td>
<td>CuSO₄</td>
<td>H₂O/t-BuOH</td>
<td>24 h</td>
<td>58%</td>
</tr>
<tr>
<td>75</td>
<td>80</td>
<td>Cu(MeCN)₄BF₄</td>
<td>DMF</td>
<td>10 h</td>
<td>78%</td>
</tr>
<tr>
<td>54</td>
<td>80</td>
<td>Cu(MeCN)₄BF₄</td>
<td>DMF</td>
<td>10 h</td>
<td>80%</td>
</tr>
</tbody>
</table>

Table 2.4 – Summary of attempted CuAAC coupling conditions. All reactions with TBTA as ligand, and monitored by LC-MS.

The remainder of the affinity probes were synthesised using Cu(MeCN)₄BF₄ as the copper(I) source and the results are summarised in Figure 2.7.
Figure 2.7 – Synthesis of ANS affinity probes by CuAAC coupling. Reagents and conditions: a) propargyl ANS 80a, CuSO$_4$ (10 mol%), sodium ascorbate, TBTA, H$_2$O/t-BuOH, rt, 18 h: 89, 59%; 92, 58%; b) propargyl ANS 80a, Cu(MeCN)$_4$BF$_4$ (10 mol%), TBTA, DMF, rt, 10 h: 89, 78%; 90, 75%; 91, 72%; 92, 80%.

2.3 Discussion

A biotinylated anisomycin affinity probe library has been synthesised in excellent yield (compounds 89-92, 58-80%) using a CuAAC coupling strategy with propargyl anisomycin 80a. The biotinylated ANS affinity probes should retain activity in affinity
pull-down experiments (Chapter 4), as previous work has shown that $N$-linked ANS molecular probes retain JNK activation.

Solubility issues were encountered initially in the CuAAC approach due to the affinity linker toolbox being sparingly soluble in $\text{H}_2\text{O}/t\text{-BuOH}$. This was remedied by changing the solvent to DMF, and the catalyst to one which is soluble in this solvent $[\text{Cu(MeCN)}_4\text{BF}_4]$. Using this catalyst, which is a stable copper(I) source, means that sodium ascorbate is no longer required. This change in solvent and catalyst increased the yields substantially and decreased the reaction time.

Although the syntheses of the linkers are quite lengthy (Table 1.1), this series of compounds shows that they can be readily applied to CuAAC click-functionalised derivatives of a natural product using a one-step protocol.
3.1 Introduction

3.1.1 Sphingolipids

Sphingolipids (SLs) are a family of membrane components found in all eukaryotes, originally thought to be little more than structural elements of the cell. At present, SLs and their metabolites are known to influence a wide range of fundamental cellular functions, such as intra- and extra-cellular signalling, proliferation and cell cycle regulation. The de novo SL biosynthesis and metabolism relevant to this chapter is depicted in Figure 3.1. Sphingolipid biosynthesis begins in the endoplasmic reticulum (ER) with the serine palmitoyltransferase (SPT) catalysed condensation of L-serine with palmitoyl coenzyme A.

Figure 3.1 – Partial pathways of sphingolipid metabolism and biosynthesis, adapted from Ogretmen et al.. Ceramide can be formed de novo (purple) or by hydrolysis of sphingomyelin (green). Ceramide is metabolised (blue) by ceramidases (CDases) to give sphingosine which in turn is phosphorylated by sphingosine kinases (SKs) to generate sphingosine-1-phosphate (S1P). SPT, serine palmitoyltransferase; SMS, sphingomyelin synthase; SMase, sphingomyelinase; CDase, ceramidase; CS, ceramide synthase; SK, sphingosine kinase; S1PP, S1P phosphatase.
Due to the vast array of cellular processes that are influenced by SLs and their metabolites, it comes as no surprise that they have been implicated in a variety of diseases, such as multiple sclerosis, asthma and cancer,\textsuperscript{147-151} and altered levels of SLs have been observed in patients of acquired diseases as well as inherited SL metabolism defects.\textsuperscript{150} Thus control of SL production has been a target for pharmaceutical therapy.

### 3.1.2 Fingolimod

**Introduction**

Myriocin (ISP-1) \textsuperscript{95}, a sphingosine-like natural product, was first isolated by Kluepfel \textit{et al.} in 1972, and was characterised as an antifungal secondary metabolite of thermophilic mould \textit{Myriococcus albomyces}.\textsuperscript{152} A screening study of \textit{Isaria} and \textit{Trichoderma} fungi by Fujita \textit{et al.}\textsuperscript{153} for immunosuppressive activity on mouse allogeneic mixed lymphocyte reaction (MLR), isolated ISP-1 from \textit{Isaria Sinclairii}, an entomopathogenic fungus. The fungus is an imperfect stage of \textit{Cordyceps Sinclairii}, and is closely related to a fungus used in Chinese traditional medicine believed to impart eternal youth.\textsuperscript{153-155} Myriocin is a potent immunosuppressant (MLR assay; IC\textsubscript{50} = 3 to 8 nM), induces apoptosis in a murine cytotoxic T-lymphocyte cell line, and inhibits SPT activity.\textsuperscript{36,156} However, compared to cyclosporin A, ISP-1 is more toxic and is less soluble.\textsuperscript{157} With the goal of improving the biological properties, SAR analysis was carried out for ISP-1 \textbf{95} (Figure 3.2), which guided the synthesis of a library of lead compounds.
Figure 3.2 – SAR analysis of myriocin (ISP-1, 95) and FTY720, 96.

Removal of the hydroxyl chiral centres at the 3- and 4- positions had no detrimental effect on potency and toxicity, as did further simplification of the polar head group which was modified to be symmetrical via reduction of the carboxylic acid into the hydroxymethyl group, which decreased toxicity by approximately 30-fold. Thus, simplifying the polar head group gives the 2-alkyl-2-amino-1,3-propanediol skeleton, which is the minimum requirement for immunosuppressant activity. Fujita had remarked in an earlier publication that side chain functionalities such as the ketone and alkene were not always necessary for biological activity; removal of the ketone or reduction to a hydroxyl moiety were tolerated, and though reduction of the alkene decreased toxicity it also substantially decreased the potency. Thus, the final modification that was made was the introduction of an aromatic moiety in the place of the alkene to restrict conformation and retain amphiphilicity, leading to the synthetic analogue fingolimod (FTY720, 96). The MLR assay of FTY720 showed an IC\textsubscript{50} value (6.1 nM), which was equivalent to that of ISP-1.

**Biological mode of action – immunomodulation**

FTY720 does not inhibit SPT activity, which suggests that its immunosuppressive activity is not equivalent to ISP-1. FTY720 acts as an immunosuppressant due to its close structural homology to sphingosine 98, a sphingolipid metabolite of the cell constituent ceramide (Figure 3.3).
Figure 3.3 – Phosphorylation via Sphingosine kinase 2. In normal cell function, SK2 converts sphingosine 98 into sphingosine-1-phosphate 97. The drug FTY720 96 is converted into (S)-FTY720-P 99 by the same enzyme.

The cellular immunomodulatory mechanism of fingolimod has been described recently in a review by Brinkmann et al. In summary, phosphorylation of sphingosine by both isoforms of SK(1 or 2) to sphingosine-1-phosphate (S1P) initiates the activation cascade of B- and T-cells, where S1P activates the G-protein coupled receptors (GPCRs), denoted as S1P<sub>1-5</sub>, a process which is necessary for the body’s release of lymphocytes from the lymph nodes to the blood. The orally available prodrug fingolimod becomes active in vivo after mono-phosphorylation by SK2, it then binds to four of the five extracellular GPCRs S1P<sub>1,3-5</sub> (which recognise S1P) inducing rapid polyubiquitination, endocytosis and proteosomal degradation of the receptors and thus preventing the release of lymphocytes from lymphoid tissue (Figure 3.4).
Figure 3.4 – Biological mode of action of S1P and FTY720, adapted from Rivera et al.\textsuperscript{163} The level of sphingosine-1-phosphate (S1P) in lymphoid tissues is normally relatively low compared with the lymph, thereby forming an S1P gradient (shaded green). S1P receptor 1 (S1PR1) expressed on T cells is responsive to the S1P gradient and promotes T-cell egress from the lymphoid organ through the endothelial barrier into lymph. S1PR1 expression is decreased after activation of the T cell in the lymphoid organ by encountering an antigen-expressing dendritic cell or by type I interferon stimulation. Effector T cells eventually re-express S1PR1 and thereby egress from the lymph node to the lymph and into the peripheral tissues. In the presence of synthetic S1PR1 ligands such as FTY720, T-cell egress might be blocked by several possible mechanisms: dissipation of the S1P gradient, downmodulation of S1PR1 on T cells by ligand-induced internalization and S1PR1-mediated closure of egress ports on the endothelium by enhancement of junctional contacts.
Fingolimod and multiple sclerosis

Though originally designed as a novel immunosuppressant, FTY720 has many other prospective uses.\textsuperscript{153} Fingolimod has recently been approved (FDA, 2010) as a new drug for the treatment of multiple sclerosis (MS). MS is a chronic autoimmune disorder of the central nervous system (CNS), which affects over 100,000 people in the UK (in a 3:1 female to male ratio), and each year 5,000 people are newly diagnosed with the disease.\textsuperscript{164,165} MS occurs when T- and B-cells cross the blood brain barrier (BBB), progress into the CNS and attack healthy cells.\textsuperscript{166} Fingolimod has shown unprecedented efficacy for reducing annual relapse rates and symptoms of MS (Table 3.1).\textsuperscript{149,167}

<table>
<thead>
<tr>
<th>Drug</th>
<th>Trade name (company)</th>
<th>Type</th>
<th>Route</th>
<th>Reduction in relapse rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon β-1a</td>
<td>Avonex (Biogen Idec)</td>
<td>Cytokine</td>
<td>IM</td>
<td>18-32%</td>
</tr>
<tr>
<td></td>
<td>Betaseron (Bayer)</td>
<td></td>
<td>SQ</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td>Extavia (Novartis AG)</td>
<td></td>
<td>SQ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rebif (Merck KGaA)</td>
<td></td>
<td>SQ</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SQ</td>
<td></td>
</tr>
<tr>
<td>Fingolimod</td>
<td>Gilenya (Novartis AG)</td>
<td>Small molecule</td>
<td>Oral</td>
<td>80-83%</td>
</tr>
<tr>
<td>Glatiramer acetate</td>
<td>Copaxone (Teva)</td>
<td>Random polymer of 4 amino acids (K,A,Y,E)</td>
<td>SQ</td>
<td>29%</td>
</tr>
<tr>
<td>Natalizumab</td>
<td>Tysabri (Biogen Idec)</td>
<td>Humanised monoclonal antibody</td>
<td>IV</td>
<td>68%</td>
</tr>
</tbody>
</table>

Table 3.1 – Comparison of MS drugs currently on market. IM – intramuscular; SQ – subcutaneous; IV – intravenous.

A recent review by Brinkmann \textit{et al} details at length the biological mode of action of FTY720 with regards to MS.\textsuperscript{149} In brief, due to FTY720’s ability to prevent egress of the T- and B-cells from lymph tissue into the blood, these cells are not able to progress into the CNS and attack nerve cells in the brain and spinal cord (Figure 3.4).
Fingolimod and cancer

Considerable evidence now exists that both SK and S1P play key roles in cancer progression, as reviewed by Pyne and Pyne and Ogretmen and Hannun. SK1 features in two major hallmarks of cancer: enhanced proliferation and metastasis/invasion. Elevated levels of the expression of the oncogene SK in mRNA transcript which encodes for the protein SK and/or the protein SK are seen across an array of cancers, including: stomach, brain, colon, and breast. In some cases, elevated levels of SK has shown to correlate with disease progression and reduced patient survival. Probably the most widely known sphingolipid, S1P is a known regulator of growth, survival and migration of mammalian cells. Cells maintain a dynamic balance of distinct sphingolipid metabolites, a ceramide-sphingosine-S1P rheostat, where the balance of the relative amounts of each of these lipids can determine cell fate. Ceramide and sphingosine are pro-apoptotic, whereas S1P promotes cell survival, thus the interconversion of the lipids directs the cell towards apoptosis or survival (Figure 3.5). Therapies which decrease the activity of SK and adjust the rheostat towards ceramide/sphingosine, thus biasing cell signalling towards apoptosis, are required for the treatment of cancer.

Figure 3.5 – The ceramide-sphingosine-S1P rheostat.

One way of targeting the S1P pathway is to use S1P receptor-selective antagonistic drugs, in particular the receptors S1P1 and S1P3 due to their roles cell proliferation, migration, invasion, and angiogenesis. A known antagonist of these receptors is FTY720 and phosphorylation of FTY720 by SK2 is crucial for its anti-MS function via immune suppression. FTY720 has been shown to possess anti-cancer properties
in various human cancer cell lines, but the mechanisms underlying the observed anti-cancer effect of FTY720 on these cancer cell lines are complex and have yet to be fully elucidated and whether the anti-cancer actions of FTY720 are regulated by phosphorylation is unclear. Many signalling pathways have been considered to explain the ability of the drug to induce apoptosis, including those arbitrated by MAPKs, phosphatase 2A inhibition, S1P receptors and Bcl-2 levels.

In synthesising an affinity probe toolbox (as seen for ANS in Chapter 2) using a FTY720 analogue and the affinity linkers developed in Chapter 1, it is hoped that the biological mechanism of action may be further clarified (in Chapter 4).
3.2 Results

3.2.1 Literature precedent

The target of this synthesis was the alkynyl FTY720 analogue 100 for use in click chemistry attachment to the affinity linker toolbox, as investigated previously with the natural product anisomycin. This click approach was used, as literature precedent exists for modification of the terminal position of FTY720 with retention of biological activity. Li et al.\textsuperscript{187} coupled an FTY720 azido-analogue 101 with an alkynyl BODIPY molecule using CuAAC chemistry to synthesise a fluorescent probe 102 (Figure 3.6).

The fluorescent probe 102 was used to visualise probe accumulation within subcellular compartments of living cells by fluorescent microscopy, and thus ascertain whether FTY720 affects cholesterol accumulation in human cells and tissues, as cellular sphingolipid and cholesterol metabolism are closely related.\textsuperscript{188}

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{figure36.png}
\caption{Structure of proposed alkynyl fingolimod and fluorescent BODIPY-fingolimod molecular probe.}
\end{figure}

Synthesis of the polar head group of the desired alkynyl fingolimod analogue 100 has been adapted from two published syntheses by the Hori group\textsuperscript{189} and the Adachi group.\textsuperscript{190} The Hori group synthesis of key intermediate triflate 107 began with commercially available \(p\)-hydroxyphenylethanol 103 as shown in Figure 3.7, and was completed in an overall yield of 32\% over 10 steps.
Figure 3.7 – Hori group synthesis of a fingolimod head group. Reagents and conditions: (a) NaHSO₃•SiO₂, n-hexane, EtOAc, reflux, 24 h; (b) TBSCI, imidazole, CH₂Cl₂, rt, 15 min; (c) LiAlH₄, THF, rt, 15 min, 92% over 3 steps; (d) imidazole, PPh₃, l₂, CH₂Cl₂, rt, 1.5 h; (e) AcNH(CO₂Et)₂, NaH, DMF, rt, 4 h, 87% over 2 steps; (f) LiAlH₄, THF, rt, 6 h; (g) Ac₂O, Et₃N, DMAP, THF, rt, 15 min, 69% over 2 steps; (h) (n-Bu)₄NF, THF, rt, 20 min; (i) Tf₂O, pyridine, CH₂Cl₂, 0 °C – rt, 15 min, 98% over 2 steps; (j) TMSC≡CH, Et₃N, PdCl₂(PPh₃)₂, CH₃CN, reflux, 1.5 h, 61%.

The Adachi group synthesis begins with the commercially available 2-(4-bromophenyl) ethanol 109, and the synthesis proceeds to the key intermediate bromide 112 as shown in Figure 318, with an overall yield of 33% over 5 steps. This synthesis achieves a similar overall yield to the previous Hori group synthesis, but in less synthetic steps, due to the alternative starting material.

Figure 3.8 – Adachi group synthesis of fingolimod head group. Reagents and conditions: (a) MsCl, Et₃N, CH₂Cl₂, rt, 2 h; (b) NaI, 2-butanone, reflux, 4.5 h, 91% over 2 steps; (c) AcNH(CO₂Et)₂, NaH, DMF, 60 °C, 3.75 h, 78%; (d) NaBH₄, CaCl₂, EtOH/H₂O, rt, ~18 h; (e) Ac₂O, pyridine, rt, ~18 h, 46% over 2 steps.¹⁹⁰

3.2.2 Fingolimod analogue synthesis

Drawing on previous syntheses of fingolimod by the Hori group¹⁸⁹ and the Adachi group,¹⁹⁰ the retrosynthesis of the fingolimod alkyne 100 was designed as shown below (Figure 3.9).
3.2.3 Polar head group synthesis

Aryl bromide

In the forward synthesis, the commercially available 2-(4-bromophenyl)ethanol 109, was iodininated via mesylate 109a as in the Adachi synthesis to furnish the iodide 110 in 78% yield. Using an Appel-like reaction, as seen in the Hori synthesis, the iodide 110 was synthesised in much greater yield (99%). The ethyl iodide 110 was condensed with diethyl acetamidomalonate to give the diester 111. The ester groups of 111 were reduced using LiAlH₄ in accordance with the Hori synthesis, though instead of the reported fully deprotected diol amine, the undesired N-ethyl diol 111a was isolated as the major product (Figure 3.10). Consequently, the reduction of the diester groups was carried out using the milder reducing agent NaBH₄ in K₂HPO₄; to give diol 111b, that was subsequently acetylated to give the corresponding bromo triacetate 112 (Figure 3.10).
Aryl iodide compounds often have greater reactivity in Pd-coupling reactions than their bromide counterparts, thus synthesis of the iodo equivalent of the bromo-aryl head group 112 was targeted.\(^{191}\) Attempts were made using the aromatic variant of the Finkelstein reaction\(^{192}\) to convert the aryl bromide 112 directly into the corresponding iodide, however the yields were unsatisfactorily low. As an alternative route, non-commercial aryl iodide 114 was targeted as the starting material for the polar head group synthesis. Sandmeyer reaction on the commercially available 4-aminophenethyl alcohol 113, converted the aniline group into the diazonium salt and, following substitution with potassium iodide, gave the aryl iodide 114 (Figure 3.11).
Figure 3.11 – Synthesis of intermediate aryl iodide 116. Reagents and conditions: a) i. NaNO₂, H₂SO₄, H₂O, 0 °C to rt, 1 h ii. KI, H₂O, rt to 60 °C, 2.5 h, 88%; b) I₂, PPh₃, DCM, rt, 2 h, 89%; c) diethyl acetamidomaleonate, NaH, DMF, rt to 50 °C, 5 h, 90%; d) NaBH₄, K₂HPO₄, EtOH, rt, 18 h, 95%; e) Ac₂O, DMAP, TEA, THF, rt, 18 h 94%.

The subsequent synthetic steps are identical to those used in the aryl bromide 112 synthesis, however a problem was encountered in the condensation reaction with diethyl acetamidomalonate which was not previously seen in the aryl bromide synthesis. During the nucleophilic substitution, diester 115 is given as the major product and styrene 115a as a side product (10-40%) by elimination, the styrene can be recovered by hydroboration to give alcohol 114. Diester 115 was then reduced and acetylated using conditions as previously mentioned in good overall yield (63%, over 5 steps) to give the iodo Sonogashira precursor 116 (Figure 3.11).

3.2.4 Sonogashira synthesis of fingolimod alkyne

The first route to be explored for the synthesis of fingolimod alkyne employed a Sonogashira coupling of aryl bromide 112 to a terminal alkyne, followed by reduction of the resultant internal alkyne and subsequent elaboration to a terminal alkyne. 6-Heptyn-1-ol 118 was chosen as the substrate for the Sonogashira coupling which was synthesised from commercially available 6-heptynoic acid 117. Following a protocol from Scheufler et al., heptynoic acid 117 was reduced to the corresponding heptynol 118 using LiAlH₄ in good yield (Figure 3.12, 89%).
The next step was the Sonogashira coupling of aryl bromide 112 and 6-heptyn-1-ol 118, using reaction conditions from the Hori synthesis. The Sonogashira reaction between aryl bromide 112 and alkyne 118 was attempted in an effort to yield the key intermediate alcohol 119 (Figure 3.13). Disappointingly, regardless of catalyst loading (5-20 mol%), solvent (THF, NEt₃) or temperature (rt, 40 °C, 80 °C), unreacted starting material was the major component observed in the ¹H NMR of the crude reaction mixture with no conditions giving an acceptable yield.

However, gratifyingly the Sonogashira coupling with the iodo-functionalised polar head group proceeded in excellent yield to give the alcohol 119, which was then reduced by hydrogenation to give alkyl alcohol 120 in quantitative yield (Figure 3.14).

The final stage in the synthesis was the introduction of the terminal alkynyl moiety for click attachment to the affinity linker toolbox. A two-step process for this was employed via oxidation of alcohol 120 and a one-carbon homologation to give the alkyne 100. Several oxidation conditions were considered, although chromium-based reagents were avoided due to the potential for contamination of the final product with biologically toxic chromium. Attempts were made using the Parikh-Doering oxidation.
and Dess-Martin periodinane (DMP) to synthesise the aldehyde 121, and both oxidation approaches gave comparable conversion to the aldehyde 121 by $^1$H NMR (87% and 82% respectively), though the Parikh-Doering oxidation went to completion in much less time.

![Figure 3.15](image1.png)  
**Figure 3.15** – Synthesis of aldehyde 121. Reagents and conditions: a) DMP, NaHCO$_3$, DCM, DMSO, rt, 18 h, 82%; b) Et$_3$N, SO$_3$•py, DMSO, rt, 2 h, 87%.

From the aldehyde 121, the terminal alkynyl moiety can be introduced either by Corey-Fuchs reaction or by Seyferth-Gilbert homologation.$^{198,199}$ The preferred method was the Ohira-Bestmann (OB) modification of the Seyferth-Gilbert homologation$^{200}$ due to the milder reaction conditions, and the reaction tolerance for crude aldehyde formed by both the Parikh-Doering and DMP oxidation. Reaction with the OB reagent 122 gave 123 and 124, as a mixture of protected and partially deprotected alkyne products as a result of the base lability of the acetate groups. However, the mixture of products could be combined and fully deprotected by hydrolysis to give fingolimod-alkyne 100 in good overall yield (Figure 3.16).

![Figure 3.16](image2.png)  
**Figure 3.16** – Synthesis of fingolimod 100. Reagents and conditions: a) Ohira Bestmann reagent, K$_2$CO$_3$, MeOH, rt, 18 h, 75% (over 2 steps from alcohol 120); b) LiOH$\cdot$H$_2$O, MeOH, 50 °C, 5 h, quant.
In fact, the oxidation/OB/hydrolysis reactions gave an improved yield of fingolimod alkyne when telescoped without any intermediary purification steps (with purification – 65%, without purification – 78%). Altogether, synthesis via the iodo polar head group gave the target fingolimod alkyne 100 in 10 steps and 31% overall yield.

3.2.5 Zipper /Negishi synthesis of fingolimod alkyne

Upon further literature research, another method came to light for installing a terminal alkyne, by isomerisation of an internal alkyne by way of an alkyne zipper reaction. An initial attempt to incorporate the zipper reaction in the synthesis was carried out by using internal alkyne 125, synthesised by Sonogashira coupling of aryl iodide 116 and 1-octyne (Figure 3.17), but no product was seen by ¹H NMR/ESI-MS of the crude reaction mixture. Instead the major product isolated was diol 127 (90% yield), where both protected hydroxyls have been revealed due to the basic conditions of the zipper reaction.

![Figure 3.17 – Alkyne zipper reaction on conjugated aryl alkyne 125. Reagents and conditions: (a) 1-octyne, PdCl₂(PPh₃)₂, Cul, Et₃N, THF, 40 °C, 18 h, 74%; (b) NaH, diaminopropane, 70 °C, 18 h; 123, 0%; 127, 90%.

Considering the proposed mechanism for the zipper reaction, it is likely that this reaction was not successful due to the absence of a CH/CH₂ on both sides of the alkyne.

The zipper reaction takes place in a basic environment, which is not compatible with the protecting group strategy on the aryl iodide 116. Thus, a second approach using the zipper reaction prior to the Negishi coupling was envisioned using commercially
available internal alkyne 128, which was converted into the terminal alkyne 129. The alkyne 129 was TMS-protected and converted to the iodide to give TMS-alkynyl iodide 130, which was used in a copper(I) iodide mediated Negishi coupling, with the more reactive iodo polar head group (Figure 3.18). The Negishi product 131 was fully deprotected using potassium carbonate to give fingolimod alkyne 100 in good yield.

Figure 3.18 – Alkyne zipper reaction. Reagents and conditions: (a) NaH, diaminopropane, 70 °C, 18 h, 62%; (b) BuLi, TMS-Cl, ; (c) PPh₃, I₂, imidazole, DCM, rt, 2 h, 95%; (d) 130, Zn, PdCl₂(dppf), Cul, DMA, 80 °C, 82%; (e) K₂CO₃, MeOH, 50 °C, 18 h, quant.

**Summary - Approaches to fingolimod alkyne**

The overall synthesis via Sonogashira coupling with the iodo polar head group gave fingolimod alkyne 100 in 10 steps and 31% overall yield, and from Negishi coupling gave fingolimod alkyne 100 in 8 steps and 32% overall yield. Both methodologies have similar overall yields, though the Negishi approach has less synthetic steps, and costs less overall in terms of reagents and starting materials used (Ohira Bestmann reagent from Sigma Aldrich; 10 cm³, ~10% in acetonitrile, £233.50). However the Sonogashira final steps (hydrogenation/oxidation/OB/hydrolysis) require little purification, and so is the preferred method.

**3.2.6 Affinity probe synthesis**

Using the same strategy as seen in Chapter 2, fingolimod alkyne was coupled to the affinity linker toolbox using a CuAAC click protocol, to generate a series of probes for use in affinity pull-down experiments (Figure 3.20). All probes were synthesised
using Cu(MeCN)$_4$BF$_4$ mediated CuAAC coupling in excellent yields, as summarised in Figure 3.20.
Figure 3.20 – Synthesis of fingolimod affinity probes. Reagents and conditions: a) Fingolimod alkyne 100, Cu(MeCN)₄BF₄, TBTA, DMF, rt, 18 h; 137, 75%; 138, 73%; 139, 78%; 140, 82%; 141, 72%; 142, 71%

3.3 Discussion

A biotinylated fingolimod affinity probe library has been synthesised in excellent yields (compounds 137 – 142, 71-82%) using a CuAAc coupling strategy with fingolimod alkyne 100, which was synthesised using Sonogashira (31% overall yield) or Negishi (32% overall yield) based coupling.

The synthesis of fingolimod alkyne 100 via the Sonogashira coupling was relatively straightforward, with all synthetic steps achieved in good to moderate yields. The only complication of the synthesis arose in the failure of the Sonogashira coupling of the bromo polar head group 112. The synthesis via the Negishi/Zipper reaction was, in comparison, much more complicated; the Zipper reaction was very capricious, and the reagents used were hazardous and not as user friendly.

Although the syntheses of the linkers are quite lengthy (Table 1.1), this series of compounds shows that they can also be readily applied to click-functionalised derivatives of a small molecule of interest using a one-step protocol as seen in Chapter 2.
Chapter 4 Biological Studies

4.1 Anisomycin

4.1.1 Ribosomal Binding Study

Preliminary biological studies were carried out at NUI Galway (with Dr H Fearnhead), to assess the potential of the ANS affinity probes in determining the cellular target of ANS in the TNBC cell lines of interest. Current data for the activity of ANS suggests that at high concentrations it acts as a peptidyl transferase inhibitor, binding to the 60S ribosomal subunit in eukaryotes, while at nanomolar concentrations it affects the MAPK pathways. To investigate protein synthesis inhibition by ribotoxic stress both in vivo and in vitro due to peptidyl transferase inhibition, a ribosomal purification was derived from a protocol by Belin et al. (Figure 4.1). The ribosomal quality was then investigated using Western Blotting.

Figure 4.1 – Protocol for the purification of ribosomes from human cell lines.
The Western Blot (also known as the protein immunoblot) is a commonly used bioanalytical technique used to detect specific proteins in the given sample of tissue homogenate or cell extract. Gel electrophoresis (SDS-PAGE) is used to separate native proteins by their 3-D structure or denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed using antibodies specific to the target protein.

A Bradford assay was carried out to analyse the total protein content of each fraction from the ribosomal purification, these were diluted to a standard concentration matched to the ribosomal fraction (0.25 µg mL\(^{-1}\)). The SDS-PAGE was run using the samples (30 µL, 7.5 µg) which had been denatured (using a Laemmli/DTT solution) to remove secondary and tertiary structure, thus allowing separation of the different proteins by molecular weight (Figure 4.2).

![Figure 4.2 – SDS page experiments for ribosomal preparation. The first lane (M) contains the BioRad molecular marker; the second lane (A) is from the whole cell lysate; the third lane (B) is the post-nuclear fraction; the fourth lane (C) is the mitochondrial fraction; the fifth lane (D) is the post-mitochondrial fraction; and the last lane (E) is the ribosomal fraction. The gel was stained with Coomassie blue dye.](image-url)
The next step was to transfer the proteins from the gel onto a PVDF membrane, in order to make the proteins accessible for antibody detection; the transfer was carried out by electroblotting. After the transfer, the membrane was blocked using milk solution to prevent non-specific binding. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

**Figure 4.3** – Luminescence and fluorescence of ribosomal protein by immunoblotting.

The primary antibody used was specific to the ribosomal protein S3 (FL-243), and the secondary antibody was linked to horseradish peroxidase (HRP) in order to create fluorescence in proportion to the amount of ribosomal protein. Figure 4.3 above shows that the ribosomal fraction (Figure 4.3; lane E) does indeed hold a high percentage of ribosomes, thus confirming that the ribosomes prepared by our protocol were of good quality and the assay conditions were appropriate.

To test the theory of protein synthesis inhibition, the biotinylated ANS affinity tags (89 and 92, Figure 4.4) were incubated with the ribosomes made through this protocol, and their binding affinity was measured using a fluorescence competition assay against ANS. The assay used SAv conjugated to horseradish peroxidase (HRP), which binds to the biotinylated affinity tags. On reaction with a chemiluminescent peroxidase substrate (luminiol) and a strong oxidising agent (hydrogen peroxide), excited
intermediates are formed. These intermediates decay to a lower energy level and return to their stable ground state, releasing a strong blue fluorescent emission (450 nm) which can be quantified by fluorimetry.

**Figure 4.4 – Principles of the fluorescence assay.** The ribosomes (R) are incubated with biotinylated affinity tag (89 and/or 92) and the competition anisomycin (ANS), and then unbound species are removed by centrifugation.

The background fluorescence was measured using the absence of ANS in the assay conditions, and also using the biotinylated affinity linker azides (Section 1.5.2; Figure 1.25; 53, 75). In the presence of ANS, it was hypothesised ANS should create a ~ 10-fold reduction in fluorescence due to competitive binding. Each assay condition was carried out in triplicate to obtain a mean fluorescence. In the competition assay between the biotin spacer ANS (Figure 4.4; 89) and anisomycin; the mean background fluorescence was 13.49 ± 4.1, and the mean competition fluorescence was 12.73 ± 2.8. For the biotin linker ANS (Figure 4.4; 92) species; the mean background fluorescence was 10.86 ± 2.1, and the mean competition fluorescence was 10.76 ± 0.7. Both results show that no competition between the biotinylated affinity tags and ANS is seen, as
both sets of results fall within the standard deviation of the triplicate mean, and no reduction of fluorescence is seen. This could mean one of two things:

1. No binding to ribosomes were seen.
2. No competition is seen due to orthogonal binding sites for the ANS and the affinity probe.

Structural data suggests that biotin linked ANS will not bind to the ribosome – as the pyrrolidine modification point of ANS plays a role in ribosomal binding. The nitrogen of the pyrrolidine group forms a hydrogen bond with N3 of C2487 in the active site crevice of the peptidyl transferase centre of the large ribosomal subunit. These data, along with previous biological work, suggests that ANS which has been modified at the pyrrolidine nitrogen has an alternative binding partner, other than the ribosome, to create a phenotypic response.
4.1.2 Affinity Pull-Down Experiments

Following the ribosomal binding study, the biotinylated ANS probe 92 was utilised in an affinity pull-down experiment with MDA-MB-468 cell lysate by Dr Fearnhead (Figure 4.15). From the initial pull-down experiment, a binding protein, PEX1, was identified, raising the possibility that targets besides the ribosome are involved in ANS-induced cell death.

![Figure 4.5](Image)

**Figure 4.5** – Pull down experimental result obtained from Dr Fearnhead, using affinity probe 92. An S-100 extract was prepared from MDA-MB-468 cells and passed over immobilized ANS. Unbound protein was washed away and specifically bound proteins eluted using free ANS and collected in 5 fractions. Proteins were resolved by SDS-PAGE and detected by Coomassie Blue staining before being identified by LC-MS.

ANS binds the ribosome and inhibits peptidyl transferase activity through ribotoxic stress\textsuperscript{118} and some have argued that this explains ANS’s anticancer activity.\textsuperscript{207} At the same time ANS also activates a ribosome binding kinase, EIF2AK2 (Protein kinase R)\textsuperscript{208} and this is reported to be required for ANS-induced apoptosis.\textsuperscript{209} EIF2AK2 may induce apoptosis by increasing JNK and p38 kinase activity, a chain of events that is called the ribotoxic stress response,\textsuperscript{120} or by phosphorylating eIF2\textalpha.\textsuperscript{210,211} However data, including our own,\textsuperscript{131} are inconsistent with a model in which an ANS-induced ribotoxic stress response or protein synthesis inhibition causes cell death.
Different mechanisms for ANS induced cell death have been proposed. These include inhibition of protein synthesis and activation of a ribotoxic stress response involving JNK and p38 and possibly EIF2AK2. Our data suggest that ANS-induced cell death does not involve protein synthesis inhibition or JNK or p38 and that alternative routes to cell death are used.

Due to Dr Fearnhead’s expertise in ANS and affinity pull-down experiments, the complete toolbox of ANS affinity probes have been sent to NUIG for further testing.
4.2 Fingolimod

4.2.1 Preliminary pull down experiments

The fingolimod affinity probes synthesised in Chapter 3 were used in a series of affinity pull-down experiments using HeLa cell lysates. The cervical cancer HeLa cell line was chosen as this cellular line has been used previously with FT720 testing. The affinity probes were incubated with Neutravidin resin; this created an activated resin with which the HeLa lysate was incubated. After overnight incubation with the HeLa lysate, the activated bead-protein mixture was washed and the isolated proteins were analysed by SDS-PAGE. The protocol for these affinity experiments is summarised in Figure 4.7.

Figure 4.7 – Protocol for affinity experiments

The first affinity experiment carried out was using compounds 137 and 140, the affinity-dependent and independent fingolimod probes. The gel from this experiment (Figure 4.8) shows that probe 137 has not pulled down specific binding proteins, whereas probe 140 has. This can be deduced since the affinity-dependent probe 137
Biological Studies

(Figure 4.8, lane E) has pulled down the same as lane B which was a blank run using the Neutravidin beads incubated with HeLa cell lysate. The protein at ~14 kDa was identified (Dr Shiran, Proteomics Core Facility, St. Andrews) as Avidin, which is a contaminant from the boiling of the beads. This result infers that the linker length of the affinity-dependent fingolimod probe is not long enough, or the biotinylated probe did not bind to the Neutravidin resin. Increasing the incubation time of the affinity probe with the resin, and of the activated affinity probe with lysate did not change this result. Lanes G and J (Figure 4.8) show that the PBS wash is efficient, as no proteins are shown in the gel.

Figure 4.8 – Legend: a) Structures of affinity probes used in the experiment 137 and 140; b) summary of experiment conducted; c) gel run: (M) SeeBlue Invitrogen molecular marker; (A) HeLa whole cell lysate; (B) pull-down with unactivated Neutravidin beads; (C) the lysate supernatant; (D) wash supernatant; (E) pull-down with beads activated with compound 137; (F) the lysate supernatant; (G) wash supernatant; (H) pull-down with beads activated with compound 140; (I) the lysate supernatant; (J) wash supernatant.

In the second series of experiments (Figure 4.9) the differences between using PBS and Triton-X in the wash steps after incubation was analysed as well as the cleavability
of the affinity probes. Two different washing procedures were used in the affinity pull-down experiments, either PBS buffer (4 x 200 µL) or PBS buffer (4 x 200 µL) followed by PBS buffer with PBS with 0.2% Triton X-100 (4 x 200 µL). Triton X-100 is a non-ionic surfactant used in biology, which was used as a stronger wash step to remove any non-specific binding proteins before the SDS-PAGE experiment.

When using the Triton-X wash, the activated beads (Triton-X wash) were incubated with slightly more lysate (150 µL instead of 100 µL) due to the increased strength of the washing step. Affinity probe 137 (Figure 4.9; lane D) has shown slight ability to pull-down binding proteins when lysate volume added is increased by 50%, but the

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**Figure 4.9** — Legend: a) Structures of affinity probes used in the experiment 137 and 140; b) summary of experiment conducted; c) gel run: (M) SeeBlue Invitrogen molecular marker; (A) HeLa whole cell lysate; (B) pull-down with unactivated Neutravidin beads; (C) pull-down with beads activated with compound 137 washed with PBS; (D) is pull-down with beads activated with compound 137 washed with PBS and Triton; (E) is pull-down with beads activated with compound 140 washed with PBS; (F) Na₂S₂O₃ cleavage supernatant of compound 140 washed with PBS; (G) the beads after cleavage with Na₂S₂O₃; (H) Na₂S₂O₃ cleavage supernatant of compound 140 washed with PBS and Triton; (I) the beads after cleavage with Na₂S₂O₃. Circled in red are the bands that were excised for analysis.
Biological Studies

Triton-X washes seem to have no effect (no loss of proteins). Affinity probe 140 has the ability to pull down a greater amount of proteins than probe 137, even after a Triton-X wash. However, it seems that the chemical cleavage of the linker did not work using the Na$_2$S$_2$O$_3$ solution concentration (0.3 M) as published previously, as no protein is evident in the supernatant lanes (Figure 4.9; lanes F, H) and the beads left after cleavage (Figure 4.9; lanes G, I) show retention of proteins. The Na$_2$S$_2$O$_3$ solution was freshly made prior to use (the longer it is left, the less efficient the cleavage), and used sparingly (the more dilute the proteins are in the supernatant).

Another two bands, in addition to the avidin band at 14 kDa, were excised and sent for analysis to Dr Shiran (Figure 4.9, bands circled in red). The highest molecular weight band at ~50 kDa was identified as Tubulin, and the band below at ~37 kDa was identified as Human Glyceraldehyde 3 phosphate dehydrogenase (GAPDH). Both proteins identified from the pull-down experiment are known for their involvement in cancer.$^{213,214}$ Further biological work is required to assess fingolimod’s interaction with these two proteins.

In the next series of experiments a comparison of the photoreactive affinity dependent probes was made (Figure 4.10: compounds 139; lanes C, D, E and 138; lanes F, G, H). Both the benzophenone- and diazirine-containing affinity probes (139 and 138 respectively) show a higher affinity and pull-down proteins with the smaller volume of lysate (100 $\mu$L), which is better than compound 137. This is potentially due to the longer chain of the affinity linker, which may prevent steric interference due to solid support, or enhance affinity to a buried binding site in a target biomolecule. The activated beads were irradiated after the proteins were bound, using a UV lamp (365 nm, 10 min) in an Eppendorf tube. Irradiation of compound 139 (Figure 4.10; lane D, E) shows an increase in low molecular weight proteins which are pulled down, in comparison to the non-irradiated compound (Figure 4.10; lane C). Compound 138 has pulled down a comparative amount of proteins; though lanes G and H are not well resolved with very weak bands of protein, and so the experiment should be repeated (as with 137) with an increased volume of lysate.
**Figure 4.10** – Legend: a) Structures of affinity probes used in the experiment 138 and 139; b) summary of experiment conducted; c) gel run: (M) SeeBlue Invitrogen molecular marker; (A) HeLa whole cell lysate; (B) pull-down with unactivated Neutravidin beads; (C) pull-down with beads activated with compound 139 washed with PBS; (D) is pull-down with beads activated with compound 139 washed with PBS and irradiated; (E) is pull-down with beads activated with compound 139 washed with PBS and Triton, and irradiated; (F) is pull-down with beads activated with compound 138 washed only with PBS; (G) is pull-down with beads activated with compound 138 washed with PBS and Triton, and irradiated; (H) is pull-down with beads activated with compound 138 washed with PBS and Triton, and irradiated.

The results of the pull-down experiments with the diazirine affinity-independent probe 141 are shown in Figure 4.11. There appears to be little difference between using just PBS washes and PBS with Triton-X (Figure 4.11; lane B and E respectively) with regards to amount of protein pulled down after irradiation. Though, as with compound
140 (Figure 4.9), the chemical cleavage of the linker has not been achieved, with many proteins left on the beads (Figure 4.11; lane D, G).

As with the previous photoreactive affinity probe, the benzophenone compound 142 was used in a series of affinity pull-down experiments (Figure 4.12). The gel shows again that there is no difference between the two different washes (PBS only, lane B;
PBS and Triton-X, lane E) with regards to protein bound after irradiation. Once more, the chemical cleavage of the linker has not been achieved, with many proteins left on the solid support (Figure 4.12; lane D, G).

From considering the results from the SDS-PAGE experiments (Figure 4.9, lane F + H; Figure 4.11, lane C + F; Figure 4.12, lane C + F), it appears as though the cleavage of the linker is not going to completion upon incubation of the beads with 0.3 M
sodium dithionite solution. The beads still have a slight orange colour to them, and the gels show that treatment of the resin with SDS after diethionite cleavage leads to the release of significant protein content. The supernatants of the linker cleavages were reduced in vacuo, and re-analysed by SDS-PAGE (Figure 4.13)

![Diagram](image)

**Figure 4.13** – Legend: a) summary of experiment conducted; b) gel run: (M) contains the BioRad precision plus dual colour standard molecular marker; (A) from Figure 4.9, lane F - Na$_2$S$_2$O$_3$ cleavage supernatant of compound 140 washed with PBS; (B) from Figure 4.9, lane H - Na$_2$S$_2$O$_3$ cleavage supernatant of compound 140 washed with PBS and Triton; (C) from Figure 4.11, lane C - Na$_2$S$_2$O$_3$ cleavage supernatant of the pull-down with beads activated with compound 141 washed only with PBS and irradiated; (D) from Figure 4.11, lane F - the Na$_2$S$_2$O$_3$ cleavage supernatant of the pull-down with beads activated with compound 141 washed with PBS and Triton; (E) from Figure 4.12, lane C - Na$_2$S$_2$O$_3$ cleavage supernatant of the pull-down with beads activated with compound 142 washed only with PBS and irradiated; (F) from Figure 4.12, lane F - the Na$_2$S$_2$O$_3$ cleavage supernatant of the pull-down with beads activated with compound 142 washed with PBS and Triton.

After concentrating the supernatants in vacuo, very faint bands of protein can be seen on the gel. These data show that the cleavage is working slightly, just not to completion using the method as previously described for the linker. Further work needs to be carried out to fully develop a cleavage protocol for these affinity linkers.
4.2.2 Western Blot

Western blotting was carried out on the photoreactive compounds to analyse efficiency of covalent biotinylation. These compounds were blotted with an anti-biotin antibody conjugated to HRP, which was used to detect biotinylated proteins in a similar protocol as previously described in Section 4.1.1.
Figure 4.14 – Legend: a) Structures of affinity probe used in the experiment 138, 139 and 141; b) summary of experiment conducted; c) Western blot: (M) SeeBlue Invitrogen molecular marker; (A) HeLa whole cell lysate; (B) from Figure 4.10, lane C - pull-down with beads activated with compound 139 washed with PBS; (C) from Figure 4.10, lane D - is pull-down with beads activated with compound 139 washed with PBS and irradiated; (D) from Figure 4.10, lane E - is pull-down with beads activated with compound 139 washed with PBS and Triton, and irradiated; (E) from Figure 4.10, lane F - is pull-down with beads activated with compound 138 washed only with PBS; (F) from Figure 4.10, lane G - is pull-down with beads activated with compound 138 washed with PBS and irradiated; (G) from Figure 4.10, lane H - is pull-down with beads activated with compound 138 washed with PBS and Triton, and irradiated; (H) from Figure 4.11, lane A - is pull-down with beads activated with compound 141 washed with PBS; (I) from Figure 4.11, lane B - is pull-down with beads activated with compound 141 washed with PBS and irradiated; (J) from Figure 4.11, lane E - is pull-down with beads activated with compound 141 washed with PBS and Triton, and irradiated.

In the Western Blot shown in Figure 4.14, lane A shows that there are no endogenously biotinylated proteins in the HeLa lysate. Furthermore, the Avidin band at around 14 kDa can be used as an internal standard for further experiments and to place the blots at the correct molecular weight.

The first three lanes of this Western Blot (Figure 4.14; lanes B, C, D) show the difference in irradiation for compound 139, the affinity-dependent biotin benzophenone fingolimod probe. The compound was not irradiated in lane B, however there are biotinylated proteins in the blot. This could be due to two things; that during the incubation of the activated beads the benzophenone photoreacted (care was taken to prevent this), or that there is a high binding affinity of the fingolimod to the binding proteins. After irradiation (Figure 4.14; lanes C, D), there are a greater number of smaller molecular weight proteins that are bound (Figure 4.14, circled in green) and the strong band at ~40 kDa disappears (Figure 4.14, circled in red). As shown in the previous SDS-PAGE of compound 139 (Figure 4.10; lanes D, E); there is no discernible difference between washing with only PBS, or PBS and Triton-X (Figure 4.14; lanes C, D).

The next three lanes of the Western Blot (Figure 4.14; lanes E, F, G) show the difference in irradiation for compound 138, the affinity-dependent biotin diazirine fingolimod probe. The compound was not irradiated in lane E (Figure 4.14), though
shows biotinylated proteins, which is the same outcome as compound 139. After irradiation (Figure 4.14; lanes F, G), there are a greater number of higher molecular weight proteins biotinylated (Figure 4.14, circled in blue); which is different to compound 139, but again the band at ~40 kDa is reduced (Figure 4.14, circled in red).

Biotinylation of proteins using compound 141, the affinity-independent diazirine fingolimod probe, is shown in lanes H, I, and J (Figure 4.14). There are very faint bands on the blot, which shows that the irradiation of the probe has not been successful. Proteins were pulled down using this probe as seen in the SDS-PAGE experiment (Figure 4.11; lanes B, E), but it appears as though the proteins were not covalently bound to the probe after irradiation of the photoreactive moiety. Perhaps a longer irradiation time (> 15 min), or use of a more transparent plastic vessel is required.

In a second series of experiments, covalent biotinylation using compound 142, the affinity-independent benzophenone fingolimod probe was investigated, along with compounds 137 and 140 as control experiments. The results of the Western blot are shown in Figure 4.15.
Figure 4.15 - Legend: a) Structures of affinity probe used in the experiment 137, 140 and 142; b) summary of experiment conducted; c) Western blot: (M) SeeBlue Invitrogen molecular marker; (A) HeLa whole cell lysate; (B) from Figure 4.12, lane A - is pull-down with beads activated with compound 142 washed with PBS; (C) from Figure 4.12, lane B - is pull-down with beads activated with compound 142 washed with PBS and irradiated; (D) from Figure 4.12, lane E - is pull-down with beads activated with compound 142 washed with PBS and Triton, and irradiated; (E) from Figure 4.9, lane C - is pull-down with beads activated with compound 137 washed with PBS; (F) from Figure 4.9, lane D - is pull-down with beads activated with compound 137 washed with PBS and Triton; (G) from Figure 4.9, lane E - is pull-down with beads activated with compound 140 washed with PBS.

As with compound 141, the bands shown on the blot (for compound 142) are very faint, and show that irradiation has not been successful. Further experiments varying the time and method of irradiation are necessary. Lanes E, F and G (Figure 4.15) were
used as controls to compare the photoreactive probes with the non-photoreactive probes. Compound 137 (Figure 4.15; lanes E, F) the biotin fingolimod probe, shows no covalent biotinylation of proteins, but compound 140 (Figure 4.15, lane G) the cleavable biotin fingolimod probe does show weak covalent biotinylation of proteins. Further work is needed to understand why this might be occurring, or to repeat the experiment to find out whether this could be a contamination effect or a one-off result.

4.2.3 Conclusion

Affinity-dependent fingolimod probes

![Chemical structures](image)

**Figure 4.16** – Structures of the affinity-dependent fingolimod probes, 137, 138, and 139.

Compound 137 failed to pull-down any proteins regardless of change in variables, such as: increase of lysate concentration, increase of incubation with cell lysate and increase of activation time (Figure 4.8 and Figure 4.9). An increase in linker length between the biotin and the ligand might enhance the ability of this type of probe to pull-down binding partners of fingolimod.

Conversely, photoaffinity probes 138 and 139, have pulled down many proteins from the cell lysate (Figure 4.10). Irradiation of the probes to create covalently bound biotinylated proteins was successful, as seen in the Western Blots (Figure 4.14). Though these experiments will need to be repeated using an increased volume of cell lysate, and varying methods of irradiation (increased time of irradiation, different
plastic vessel used), as the Western blots showed very weak bands of proteins. The photoreactive probes have shown in the Western blot experiments, that without irradiation there are biotinylated proteins, therefore these photoreactive groups react autonomously without irradiation (even though the incubations were carried out in the absence of light).

**Affinity-independent fingolimod probes**

![Figure 4.17 - Structures of the affinity-independent fingolimod probes, 140, 141, and 142.](image)

The use of compound 140 resulted in the pull-down of binding proteins from the cell lysate (Figure 4.9), three of which were identified; GAPDH, tubulin, and avidin (a contaminant from the Neutravidin® beads). Further biological work is required to comprehend how these proteins might interact with fingolimod, and if they are crucial for anti-cancer activity.

The use of compounds 141 and 142, resulted in successful pull-down of binding proteins from the HeLa cell lysate (Figure 4.11 and 4.12 respectively). Irradiation of these compounds using a UV lamp during the experiments was not as successful as the affinity-dependent linkers (Figure 4.14 and 4.15), thus an improved irradiation procedure for these compounds are required.
Chemical cleavage of the diazobenzene linker was unsuccessful with the current protocol used (Na$_2$S$_2$O$_3$, 0.3 M, 15 min), either a higher concentration or a longer cleavage time is required for these affinity-independent probes. Alternatively, with a slight modification in the structure of the diazobenzene, the addition of a phenolic hydroxyl group at the ortho-position to the diazo bond (Section 1.5.3, Figure 1.22, compound 41 for example) can decrease the cleavage time of the linker at lower salt concentrations (<100 mM).

### 4.3 Future Work

#### 4.3.1 Anisomycin

Future work using the ANS affinity probe library to further elucidate the molecular binding targets of ANS is being carried out by Dr H Fearnhead (NUIG). Hopefully with the use of the photoreactive affinity probes, low affinity or less abundant proteins can be found.

#### 4.3.2 Fingolimod

The initial pull-down and Western Blotting of the fingolimod affinity probe library has provided a helpful initial starting point for further investigations. Using the fingolimod affinity probes many proteins were pulled down from the full cell lysate. By separating the lysate into different cellular fractions, it is hoped that a more detailed overview of binding proteins could be investigated. The current cleavage protocol for the affinity-independent probes was not successful, and thus must be investigated further.
Chapter 5  Experimental

5.1 General Experimental

All non-aqueous reactions were performed using oven-dried glassware (140 °C) cooled in a desiccator prior to use, under a positive atmosphere of nitrogen or argon using anhydrous solvents, unless otherwise stated. Toluene, THF, MeOH, Et₂O, DCM, and MeCN were dried and purified by passage through activated alumina columns (Glass Contour Purification System). All starting materials and reagents were purchased from commercial suppliers and used as supplied. Aqueous solutions of acids, bases or inorganic salts are reported as: solution (volume; molarity or level of saturation).

Flash column chromatography (FCC) was carried out using powdered silica (Merck Geduran 60) of pore size 60 Å and particle size 40-63 µm, under positive pressure by hand pump. Eluent compositions are quoted as v / v ratios. Thin layer chromatography (TLC) was performed on Merck 60F₂₄₅ (0.25 mm) aluminium backed silica plates and visualised by ultraviolet light, or by using an appropriate staining solution (KMnO₄, ninhydrin, bromocresol green).

Infra-red (IR) spectra were recorded using a Shimadzu IR Affinity-1 instrument as thin films. Value of peaks at maximum absorbance (νₘₐₓ) are quoted in wavenumbers (cm⁻¹). Melting points (mp) were determined using a Gallenkamp melting point apparatus; the temperature range and whether the substance undergoes decomposition (dec.) over this range is reported.

Nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature (298 K, unless otherwise stated) on a Bruker AVA400, AVA500 or AVA600 spectrometer running at 400, 500, or 600 MHz (¹H spectra) or 101, 126, 151 Hz (¹³C spectra, respectively. Chemical shifts (δ values) are reported in parts-per-million (ppm) relative to tetramethylsilane (¹H and ¹³C spectra; δₜₐₘ₂₅ = 0) and are calibrated to the residual solvent peak. ¹H NMR data are reported as follows: chemical shift, relative intensity, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, m = multiplet, br = broad), coupling constants (J value, Hz), and interpretation.
**Experimental**

$^{13}$C NMR data are reported as follows: chemical shift, relative intensity and assignment (C = quaternary, CH = methane, CH$_2$ = methylene, CH$_3$ = methyl). $^{13}$C assignments were confirmed by DEPT135 and/or DEPT90 spectra.

Electrospray ionisation (ESI) mass spectra were performed using a Finnegan LCQ spectrometer or a Bruker micrOTOF II spectrometer at the University of Edinburgh. Mass-to-charge ratios ($m/z$) of all parent (molecular) ions ([M]$^{+/-}$) and their intensities are reported, followed by (major) fragment or adduct ions and their intensities. Calibration of spectrometer was carried out using a sodium formate solution (0.1 mg/cm$^3$ in 9:1 IPA:H$_2$O).

**Safety in the Handling of Sodium Azide and other Azides**

Sodium azide is toxic and can be absorbed through the skin. It decomposes explosively upon heating to above 275 °C. Sodium azide reacts vigorously with CS$_2$, bromine, nitric acid, dimethyl sulfate, and a series of heavy metals, including copper and lead. In reaction with water or Brønsted acids, the highly toxic and explosive hydrogen azide is released. It has been reported that sodium azide and polymer-bound azide reagents form explosive di- and triazidomethane with CH$_2$Cl$_2$ and CHCl$_3$, respectively. Heavy-metal azides that are highly explosive under pressure or shock are formed when solutions of NaN$_3$ or HN$_3$ vapours come into contact with heavy metals or their salts. Heavy-metal azides can accumulate under certain circumstances, for example, in metal pipelines and on the metal components of diverse equipment (rotary evaporators, freeze drying equipment, cooling traps, water baths, waste pipes), and thus lead to violent explosions. Some organic and other covalent azides are classified as toxic and highly explosive, and appropriate safety measures must be taken at all times.
5.2 Experimental for Chapter 1

4-[(E)-2-(4-hydroxyphenyl)diazen-1-yl]benzoic acid 48

Aminobenzoic acid 47 (1.23 g, 9.0 mmol) was dissolved in a mixture of HCl (1.2 cm³; conc.) and H₂O (24 cm³), and the suspension was cooled to 0 °C. Sodium nitrite (1.04 g, 15.0 mmol), dissolved in H₂O (5 cm³) was added dropwise to the reaction mixture and stirred for 20 min. Next, a solution of phenol (677 mg, 7.20 mmol), sodium hydroxide (257 mg, 6.42 mmol) and potassium carbonate (1.33 g, 9.60 mmol) in H₂O (20 cm³) at 0 °C was added dropwise to the reaction. The reaction was allowed to reach rt and stirred for 3 h. The dark brown suspension formed was acidified using HCl (50 cm³; 1 M), and subsequently filtered. The solid residue was solubilised in MeOH, loaded onto silica and the solvent was removed in vacuo. Column chromatography (15% MeOH in DCM) gave the product 48 as an orange solid (1.85 g, 85%). Rf (DCM:MeOH, 19:1) = 0.25; mp 273-275 °C; IR (neat, cm⁻¹) 3302 (OH), 1635 (CO), 1544 (N=N); ¹H NMR δ (500 MHz, CD₃OD) 8.19 (2H, d, J = 8.6 Hz, ArH), 7.92 (2H, d, J = 8.6 Hz, ArH), 7.90 (2H, d, J = 8.9 Hz, ArH), 6.97 (2H, d, J = 8.9 Hz, ArH); ¹³C NMR δ (126 MHz, CD₃OD) 162.87 (C), 156.75 (C), 147.61 (C), 133.52 (C), 131.75 (2 x CH), 126.40 (2 x CH), 123.15 (2 x CH), 116.85 (2 x CH); m/z (ESI+, MeCN) 243 ([M+H]⁺, 100).

All spectroscopic data were in good agreement with the literature.⁹⁰

Tert-butyl N-(2-aminoethyl)carbamate

To a stirred solution of 1,2-diaminoethane (2.49 g, 41.8 mmol) in DCM (25 cm³) was added Boc₂O (1.53 g, 7.0 mmol) in DCM (100 cm³) dropwise over 5 h. The reaction
mixture was then stirred for 24 h at rt, and then concentrated in vacuo to give a colourless oil. The oil was dissolved in DCM (50 cm$^3$) and washed with Na$_2$CO$_3$ (50 cm$^3$; 2M), the aqueous layer was extracted with DCM (2 x 50 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo to give the Boc protected amine as a colourless oil (6.4 g, 95%).

$R_f$ (DCM:MeOH, 9:1) = 0.12; IR (neat, cm$^{-1}$) 3400-3200 (NH), 1710 (CO); $^1$H NMR $\delta$ (400 MHz, CD$_3$OD) 3.10 (2H, t, $J = 6.3$ Hz, CH$_2$NHCO), 2.68 (2H, t, $J = 6.3$ Hz, CH$_2$NH$_2$), 1.44 (9H, s, 3 x CH$_3$); $^{13}$C NMR $\delta$ (126 MHz, CDCl$_3$) 156.37 (C), 79.39 (C), 43.42 (CH$_2$), 41.95 (CH$_2$), 28.53 (3 x CH$_3$); m/z (ESI+, MeCN) 161 ([M+H]$^+$, 100).

All spectroscopic data were in good agreement with the literature.$^{90}$

$N$-[2-{4-[{(E)-2-(4-hydroxyphenyl)diazen-1-yl]phenyl}} formamido)ethyl]carbamate

To a stirred solution of benzoic acid 48 (500 mg, 2.06 mmol) in DMF (15 cm$^3$) was added EDC (388 mg, 2.50 mmol) and HOBt (135 mg, 1 mmol) and stirred for 30 min. To the reaction was added $N$-Boc-ethylene diamine 642 mg, 4.00 mmol), and the reaction was stirred for 48 h. The solvent was then removed in vacuo, the crude material was dissolved in DCM (50 cm$^3$) and washed with HCl (50 cm$^3$; 1 M). The organic phase was dried (MgSO$_4$) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 49:1) to give the product as an orange solid (490 mg, 62%). $R_f$ (DCM:MeOH, 19:1) = 0.3; mp 168-172 °C; IR (neat, cm$^{-1}$) 3400-3200 (NH), 1650 (CO), 1635 (CO), 1541 (N=N); $^1$H NMR $\delta$ (500 MHz, CD$_3$OD) 7.97 (2H, d, $J = 8.5$ Hz, ArH), 7.88 (2H, d, $J = 8.5$ Hz, ArH), 7.85 (2H, d, $J = 8.8$ Hz, ArH), 6.92 (2H, d, $J = 8.8$ Hz, ArH), 3.48 (2H, dd, $J = 6.6$, 5.6 Hz, CH$_2$), 3.33 – 3.25 (2H, m, CH$_2$), 1.43 (9H, s, 3 x CH$_3$); $^{13}$C NMR $\delta$ (126 MHz, CD$_3$OD) 169.76 (C), 163.53 (C), 158.83 (C), 156.01 (C), 147.35 (C), 136.60 (C), 129.38 (2 x CH), 126.40 (2 x
CH), 123.22 (2 x CH), 117.05 (2 x CH), 80.21 (C), 41.55 (CH₂), 40.86 (CH₂), 28.75 (3 x CH₃); m/z (ESI+, MeCN) 385 ([M+H]⁺, 100).

All spectroscopic data were in good agreement with the literature.⁹⁰

*Tert-buty1 N-[2-(((4-[E]-2-[4-(3-bromoproxy)phenyl]diazen-1-yl]phenyl)formamido)ethyl]carbamate 49*

To a stirred solution of phenol 48 (480 mg, 1.25 mmol) in MeCN (18 cm³) was added K₂CO₃ (1.38 g, 10 mmol) and the reaction was heated to 70 °C. 1,3-Dibromopropane (1.02 cm³, 10 mmol) was added to the stirred solution and the reaction was heated to reflux for 5 h. The solvent was removed *in vacuo* to give the crude bromide was purified by flash chromatography (DCM:MeOH, 19:1) to give the bromide product 49 as an orange solid (405 mg, 65%). Rₓ (DCM:MeOH, 19:1) = 0.55; mp 185-189 °C; IR (neat, cm⁻¹) 3400-3200 (NH), 1702 (CO), 1650 (CO), 1541 (N=N); ¹H NMR δ (500 MHz, CDCl₃) 7.96 (2H, d, J = 8.4 Hz, ArH), 7.93 (2H, d, J = 9.0 Hz, ArH), 7.90 (2H, d, J = 8.4 Hz, ArH), 7.35 (1H, s, NH), 7.02 (2H, d, J = 9.0 Hz, ArH), 5.05 (1H, br s, NH), 4.21 (2H, t, J = 5.8 Hz, CH₂), 3.63 (2H, t, J = 6.4 Hz, CH₂), 3.61-3.56 (2H, m, CH₂), 3.47-3.38 (2H, m, CH₂), 2.37 (2H, qn, J = 6.1 Hz, CH₂), 1.44 (9H, s, 3 x CH₃); ¹³C NMR δ (126 MHz, CDCl₃) 167.29 (C), 161.73 (C), 157.82 (C), 154.56 (C), 147.29 (C), 135.49 (C), 128.13 (2 x CH), 125.23 (2 x CH), 122.73 (2 x CH), 114.96 (2 x CH), 80.27 (C), 65.85 (CH₂), 42.52 (CH₂), 40.06 (CH₂), 32.39 (CH₂), 29.85 (CH₂), 28.50 (3 x CH₃); m/z (ESI+, MeCN) 527 ([M+Na]⁺, 20%), 505 ([M+H]⁺, 40), 449 ([M–Boc+2Na]⁺, 100), 405 ([M–Boc+H]⁺, 90).

All spectroscopic data were in good agreement with the literature.⁹⁰
**Experimental**

*Tert-buty1 N-[2-({4-[(E)-2-{4-(3-azidopropoxy)phenyl]diazen-1-yl}phenyl]formamido)ethyl]carbamate 50*

To a stirred solution of bromide 49 (514 mg, 1.02 mmol) in DMF (11 cm³) was added NaN₃ (198 mg, 3.05 mmol), and the reaction was stirred overnight (18 h) at rt. The reaction was concentrated *in vacuo*, and the crude material was dissolved in Et₂O (50 cm³) and washed with H₂O (50 cm³) and brine (50 cm³, sat aq). The organic phase was dried (MgSO₄) and concentrated *in vacuo*. The residue was purified by column chromatography (DCM:MeOH, 19:1) to give the azide product 50 as an orange solid (424 mg, 89%). Rₚ (DCM:MeOH, 19:1) = 0.57; mp 169-172 °C; IR (neat, cm⁻¹) 3400-3200 (NH), 2100 (N₃), 1670 (CO), 1635 (CO), 1541 (N=N); ¹H NMR δ (601 MHz, CDCl₃) 7.99 – 7.87 (6H, m), 7.02 (2H, d, J = 9.0 Hz), 4.97 (1H, s), 4.16 (2H, t, J = 6.0 Hz), 3.59 (2H, q, J = 5.1 Hz), 3.56 (2H, t, J = 6.6 Hz), 3.44 (2H, dd, J = 10.3, 4.7 Hz), 2.11 (2H, p, J = 6.3 Hz), 1.44 (9H, s); ¹³C NMR δ (126 MHz, CDCl₃) 167.24 (C), 161.71 (C), 157.87 (C), 154.55 (C), 147.28 (C), 135.48 (C), 128.13 (2 x CH), 125.24 (2 x CH), 122.73 (2 x CH), 114.93 (2 x CH), 80.33 (C), 65.04 (CH₂), 48.31 (CH₂), 42.63 (CH₂), 40.04 (CH₂), 28.89 (CH₂), 28.50 (3 x CH₃); m/z (ESI+, MeCN) 957 ([2M+Na]⁺, 5%), 490 ([M+Na]⁺, 100), 468 ([M+H]⁺, 5), 412 ([M–Boc+2Na]⁺, 10), 368 ([M–Boc+H]⁺, 10); HRMS (ESI+, MeCN) [M+Na]⁺ found 490.2171, C₂₃H₂₉N₇O₄Na requires 490.2173.

All spectroscopic data were in good agreement with the literature.⁹⁰
Experimental

*N-(2-aminoethyl)-4-[(E)-2-[4-(3-azidopropoxy)phenyl]diazen-1-yl]benzamide hydrochloride 42*

Acetyl chloride (10 cm$^3$) was added to MeOH (60 cm$^3$) at 0 °C and the solution was stirred for 30 min. Boc-protected linker azide 50 (667 mg, 1.43 mmol) was added to the solution and stirred for 1 h. Et$_2$O (20 cm$^3$) was added and the amine hydrochloride salt precipitated as a yellow-orange solid. The salt 42 was filtered and used with no further purification (530 mg, 92%). $R_f$ (DCM:MeOH, 9:1) = 0.2; IR (neat, cm$^{-1}$) 3400-3200 (NH), 2100 (N$_3$), 1635 (CO), 1541 (N= N); $^1$H NMR $\delta$ (500 MHz, CD$_3$OD) 8.86 (1H, t, $J = 5.7$ Hz, NH), 8.07 (2H, d, $J = 8.5$ Hz, ArH), 8.01 – 7.93 (4H, m, ArH), 7.14 (2H, d, $J = 9.1$ Hz, ArH), 4.22 (2H, t, $J = 6.0$ Hz, CH$_2$), 3.73 (2H, q, $J = 5.8$ Hz, CH$_2$), 3.57 (2H, t, $J = 6.6$ Hz, CH$_2$), 2.12 (2H, p, $J = 6.4$ Hz, CH$_2$); m/z (ESI+, MeCN) 368 ([M+H]$^+$, 100%).

All spectroscopic data were in good agreement with the literature.$^{90}$

**2,5-Dioxopyrrolidin-1-yl-5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanoate 52**

Biotin 51 (500 mg, 2.05 mmol) and $N$-hydroxysuccinimide (236 mg, 2.05 mmol) were dissolved in DMF (10 cm$^3$), and to this was added DCC (553 mg, 2.68 mmol). The reaction was stirred overnight (18 h) at rt. The DCU precipitate was filtered off, and the filtrate was concentrated in vacuo. The residue was resuspended in cold hexane (50 cm$^3$), filtered and the solid was washed with cold IPA (3 x 10 cm$^3$). The solid was then recrystallised from IPA to give the product 52 as a colourless powder (651 mg,
93%). IR (neat, cm⁻¹) 3240 (NH), 1816 (CO, NHS), 1788 (CO, NHS), 1745 (CO),
1728 (CO), 1697 (CO); ¹H NMR δ (601 MHz, (CD₃)₂SO) 6.40 (1H, s, NHCHCH),
6.35 (1H, s, NHCH₂), 4.33 – 4.29 (1H, m, NHCH), 4.17 – 4.12 (1H, m,
NHCHCH₂), 3.11 (1H, dd, J = 8.2, 6.4, 4.6 Hz, NHCHCH), 2.83 (1H, dd,
J = 12.4, 5.2 Hz, CH₃H₂S), 2.82 (4H, br s, 2 x CH₂CO), 2.67 (2H, t, J = 7.4 Hz,
CH₂), 2.58 (1H, d, J = 12.4 Hz, CH₃H₂S), 1.70 – 1.61 (3H, m, SCHCH₃H₂CO), 1.55 –
1.47 (1H, m, SCHCH₂H₃), 1.46 – 1.38 (2H, m, CH₂CH₂H₂); ¹³C NMR δ (151 MHz,
(CD₃)₂SO) 170.22 (2 x C), 168.90 (C), 162.64 (C), 60.97 (CH), 59.15 (CH),
55.19 (CH), 40.37 (CH₂), 29.98 (CH₂), 27.80 (CH₂), 27.54 (CH₂), 25.42 (2 x CH₂), 24.28
(CH₂); m/z (ESI+, MeCN) 380 ([M+K]⁺, 5%), 364 ([M+Na]⁺, 45), 342 ([M+H]⁺, 100);
HRMS (ESI+, MeCN) [M+H]⁺ found 342.1111, C₁₄H₂₀N₃O₅S requires 342.1118.

All spectroscopic data were in good agreement with the literature.²¹⁵

5-[(3aS,4S,6aR)-2-Oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-[2-[(4-
[(E)-2-[(3-azidopropoxy)phenyl]diazen-1-

To a stirred solution of amino-linker-azole 42 (70.0 mg, 0.179 mmol) in 1:1
MeCN/H₂O (4 cm³), triethylamine (18.0 µL, 0.179 mmol) was added. Biotin-NHS 52
(61.3 mg, 0.179 mmol) in n-propanol (6 cm³) was added to the reaction mixture, which
was stirred for 24 h at rt. The reaction mixture was then concentrated in vacuo. The
crude material was purified by flash chromatography (DCM:MeOH, 9:1) to give the
pure product 53 as a yellow solid (75 mg, 75% yield). Rf (DCM:MeOH, 9:1) = 0.3;
IR (neat, cm⁻¹) 3302 (NH), 2092 (N₃), 1697 (CO), 1666 (CO), 1635 (CO), 1544
(N=N); ¹H NMR δ (500 MHz, (CD₃)₂SO) 8.63 (1H, t, J = 5.5 Hz, NH), 8.02 (2H, d,
J = 8.4 Hz, ArH), 7.95 – 7.91 (2H, m, NH + ArH), 7.90 (2H, d, J = 8.4 Hz, ArH), 7.87
(1H, t, J = 5.4, NH), 7.17 (2H, d, J = 9.0 Hz, ArH), 6.40 (1H, br s, NH), 6.33 (1H, br s, NH), 4.28 (1H, m, CHCH3S), 4.18 (2H, t, J = 6.1 Hz, CH2O), 4.11 – 4.06 (1H, m, CHCHS), 3.55 (2H, t, J = 6.7 Hz, CH2N3), 3.34 (2H, br dd, J = 12.4, 6.1 Hz, CH2NH), 3.25 (2H, br dd, J = 12.4, 6.4, CH2NH), 3.06 – 3.02 (1H, m, CHS), 2.79 (1H, dd, J = 12.5, 5.1 Hz, CH3H6S), 2.57 (1H, d, J = 12.5 Hz, CH3H6S), 2.08 (2H, t, J = 7.2 Hz, CH2CO), 2.03 (2H, qn, J = 6.4, CH2CH2N3), 1.63 – 1.56 (1H, m, SCHCH4H6), 1.56 – 1.48 (2H, m, CH2CH2CO), 1.48 – 1.41 (1H, m, SCHCHCH4H6), 1.36 – 1.23 (2H, m, CHCH2CH2); 13C NMR δ (126 MHz, (CD3)2SO) 172.34 (C), 165.57 (C), 162.56 (C), 161.47 (C), 153.36 (C), 146.24 (C), 135.97 (C), 128.31 (2 x CH), 124.73 (2 x CH), 121.86 (2 x CH), 115.11 (2 x CH), 65.23 (CH2), 60.92 (CH), 59.12 (CH), 55.23 (CH), 47.60 (CH2), 40.04 (CH2), 39.59 (CH2), 38.12 (CH2), 35.18 (CH2), 28.03 (CH2), 27.97 (CH2), 27.93 (CH2), 25.11 (CH2); m/z (ESI+, MeCN) 616 ([M+Na]+, 100%), 594 ([M+H]+, 100%); HRMS (ESI, +) found [M+Na]+ 616.2426, C28H35N9O4SNa requires 616.2425.

5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-2-[(4-[(E)-2-[4-(3-aminopropoxy)phenyl]diazen-1-yl]phenyl]formamido)ethyl]pentanamide 54

To a stirred solution of biotin-linker-azide 53 (200 mg, 0.337 mmol) in THF/H2O (9 cm3, 10:1) was added PPh3 (97.2 mg, 0.371 mmol). The reaction was stirred overnight (18 h) at rt. The solvent was removed in vacuo, and the crude material was purified by flash chromatography (DCM:MeOH:NH3, 80:18:2) to give the biotin-linker-amine 54 as an orange solid (126 mg, 67%). Rf (DCM:MeOH:NH3, 80:18:2) = 0.34; IR (neat, cm⁻¹) 3290 (NH), 2931 (NH), 1697 (CO), 1633 (CO), 1546 (N=N); 1H NMR δ (601 MHz, (CD3)2SO) 8.64 (1H, t, J = 5.5 Hz, NH), 8.03 (2H, d, J = 8.5 Hz, ArH), 7.95 (1H, m, NH), 7.93 (2H, d, J = 8.9 Hz, ArH), 7.89 (2H, d, J = 8.5 Hz, ArH), 7.16
(2H, d, J = 8.9 Hz, ArH), 6.39 (1H, s, NHCHCH), 6.33 (1H, s, NHCHCH2), 4.28 (1H, dd, J = 7.7, 5.1 Hz, NHCHCH2), 4.19 (2H, t, J = 6.2 Hz, OCH2), 4.09 (1H, ddd, J = 7.4, 4.6, 1.9 Hz, NHCHCH), 3.34 (2H, d, J = 6.2 Hz, CH2NH), 3.25 (2H, q, J = 6.2 Hz, CH2NH), 3.04 (1H, ddd, J = 8.5, 6.3, 4.4 Hz, NHCHCH), 2.89 – 2.83 (2H, m, CH2NH2), 2.79 (1H, dd, J = 12.4, 5.1 Hz, CHCH2H6S), 2.56 (1H, d, J = 12.4 Hz, CHCH2H6S), 2.08 (2H, t, J = 7.2 Hz, CH2CO), 1.98 – 1.92 (2H, m, OCH2CH2), 1.63 – 1.55 (1H, m, SCHCH2H6), 1.54 – 1.48 (2H, m, CH2CH2CO), 1.49 – 1.40 (1H, m, SCHCH2H6), 1.36 – 1.22 (1H, m, CHCH2CH2); 13C NMR δ (126 MHz, DMSO) 172.38 (C), 165.58 (C), 162.64 (C), 161.75 (C), 153.38 (C), 146.15 (C), 135.97 (C), 128.43 (2 x CH), 124.84 (2 x CH), 121.96 (2 x CH), 115.15 (2 x CH), 65.75 (CH2), 60.96 (CH), 59.15 (CH), 55.35 (CH), 39.69 (CH2), 39.30 (CH2), 38.15 (CH2), 37.42 (CH2), 35.24 (CH2), 29.64 (CH2), 28.14 (CH2), 28.01 (CH2), 25.22 (CH2); m/z (ESI+, MeCN) 1135 ([2M+H]+, 10%), 590 ([M+Na]+, 20), 568 ([M+H]+, 100); HRMS (ESI+, MeCN) [M+H]+ found 568.2709, C28H38N7O4S requires 568.2701.

Methyl-(2S)-6-(((tert-butoxy)carbonyl)amino)-2-(((9H-fluoren-9-ylmethoxy)carbonyl)amino)hexanoate 56

To a stirred solution of Fmoc-Lys(Boc)-OH 55 (6.68 g, 14.3 mmol) in DCM (40 cm³), was added EDC (3.01 g, 15.7 mmol), HOBT (2.12 g, 15.7 mmol) and MeOH (5.77 cm³, 1.17 mmol). The reaction mixture was stirred overnight (18 h) at rt under N2. The solvent was removed in vacuo, the residue was solubilised in DCM (50 cm³) and washed with H2O (50 cm³) and NaCl (50 cm³, sat aq). The organic layer was dried (MgSO4) and concentrated in vacuo. The residue was purified by column chromatography (EtOAc:Hexane, 1:1) to give the product 56 as a colourless foam (5.85 g, 85%). Rf (EtOAc:Hexane, 1:1) = 0.34; 1H NMR δ (500 MHz, (CD3)2SO) 7.91 (2H, d, J = 7.5 Hz, 2 x ArH), 7.75 (1H, d, J = 7.9 Hz), 7.73 (2H, dd, J = 7.4, 2.4 Hz),
Experimental

7.43 (2H, t, J = 7.4 Hz), 7.35 (2H, td, J = 7.4, 1.2 Hz), 6.77 (1H, t, J = 5.7 Hz), 4.35 – 4.27 (2H, m), 4.24 (1H, t, J = 7.1 Hz), 4.00 (1H, td, J = 8.8, 5.2 Hz), 3.63 (3H, s, OCH₃), 2.95 – 2.85 (2H, m), 1.72 – 1.57 (2H, m), 1.38 (9H, s, 3 x CH₃), 1.35 – 1.23 (4H, m); m/z (ESI+, MeCN) 505 ([M+Na]⁺, 20%), 383 ([M-Boc+H]⁺, 100).

All spectroscopic data were in good agreement with the literature.²¹⁶


To a solution of Fmoc-Lys(Boc)-OMe 56 (2.69 g, 5.58 mmol) in DCM (20 cm³) was added TFA (14.2 cm³, 83.8 mmol), and the reaction was stirred for 2 h at rt under N₂. The solvent was removed in vacuo, and the residue was dried under high vacuum (2 h) to remove residual TFA. The residue was resuspended in methanol (10 cm³), to this was added potassium carbonate (3.33 mg, 24.1 mmol), CuSO₄•5H₂O (55.8 mg, 0.223 mmol), and imidazole-1-sulfonyl azide (1.41 mg, 6.70 mmol), and the reaction was stirred for 4 h at rt in the dark. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (200 cm³), and was washed with HCl (2 x 100 cm³; 2 N aq), and brine (150 cm³), dried (MgSO₄) and concentrated in vacuo. The solid was purified by column chromatography (EtOAc:hexane, 1:1) to give the desired compound 58 as a colourless solid (1.51 g, 64%). Rᵣ (EtOAc:Hexane, 1:1) = 0.67; IR (neat, cm⁻¹) 3331 (NH), 2092 (N₃), 1716 (CO); ¹H NMR δ (500 MHz, CDCl₃) 7.77 (2H, dd, J = 7.6, 0.7 Hz, 2 x ArH), 7.60 (2H, br d, J = 4.7 Hz, 2 x ArH), 7.41 (2H, t, J = 7.5 Hz, 2 x ArH), 7.32 (2H, tt, J = 7.5, 1.2 Hz, 2 x ArH), 5.29 (1H, d, J = 8.3 Hz, NH), 4.40 (3H, m, CH₂O + NHCH), 4.23 (1H, t, J = 6.9 Hz, CHCH₂O), 3.77 (3H, s, OCH₃), 3.28 (2H, t, J = 6.6 Hz, CH₂N₃), 1.93 – 1.84 (1H, m, NHCHCH₂H₉), 1.75 – 1.66 (1H, m, NHCHCH₂H₉), 1.65 – 1.57 (2H, m, CH₂CH₂N₃), 1.50 – 1.36 (2H, m, CH₂CH₂CH₂N₃); ¹³C NMR δ (126 MHz, CDCl₃) 166.84 (C), 160.06 (C), 156.02 (C), 144.03 (C), 143.90
Experimental

(C), 141.50 (C), 127.88 (2 x CH), 127.22 (2 x CH), 125.21 (2 x CH), 120.15 (2 x CH), 67.16 (CH₂), 53.77 (CH), 52.65 (CH₃), 51.24 (CH₂), 47.35 (CH), 32.39 (CH₂), 28.54 (CH₂), 22.54 (CH₂); m/z (ESI+, MeCN) 431 ([M+Na]⁺, 100%); HRMS (ESI+, MeCN) [M+Na]⁺ found 431.1693, C₂₂H₂₄N₄O₄Na requires 431.1690.

Methyl (2S)-2-amino-6-azidohexanoate 59

To a stirred solution of Fmoc-Lys(N₃)-OMe 58 (385 mg, 0.943 mmol) in DMF (20 cm³) was added DBU (359 mg, 2.36 mmol). After 30 min the reaction was acidified with glacial acetic acid (170 mg, 2.83 mmol), and the solvent was removed in vacuo. The residue was purified by flash chromatography (DCM:MeOH, 19:1) to give the product 59 as a yellow oil (166 mg, 95%). IR (neat, cm⁻¹) 2092 (N₃); ¹H NMR δ (500 MHz, CDCl₃) 3.73 (3H, s, OC₃H₃), 3.51 – 3.42 (1H, m, NH₂CH), 3.28 (2H, t, J = 6.8 Hz, CH₂N₃), 1.82 – 1.71 (1H, m, CHCH₃HₐHₖ), 1.68 – 1.54 (3H, m, CHCH₃Hₐ + CH₂CH₃N₃), 1.55 – 1.40 (2H, m, CHCH₂CH₂); ¹³C NMR δ (126 MHz, CDCl₃) 176.37 (C), 54.28 (CH), 52.18 (CH₃), 51.35 (CH₂), 34.39 (CH₂), 28.76 (CH₂), 23.02 (CH₂); m/z (ESI+, MeCN) 209 ([M+Na]⁺, 20%).

All spectroscopic data were in good agreement with the literature.²¹⁷
Experimental

Methyl-(2S)-2-amino-6-{{(tert-butoxy)carbonyl}amino}hexanoate 60

To Fmoc-Lys(Boc)-OMe 56 (2.48 g, 5.14 mmol) was added 20% piperidine in DMF (25 cm³, 0.506 mol) and the reaction was stirred for 3 h at rt. The solvent was removed in vacuo and the residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 60 as a yellow oil (1.20 g, 89%). Rf (DCM:MeOH, 9:1) = 0.3; 1H NMR δ (500 MHz, MeOD) 3.72 (3H, s, OCH₃), 3.43 (1H, dd, J = 7.0, 5.9 Hz, NH₂CH), 3.03 (2H, t, J = 6.9 Hz, CH₂NH), 1.77 – 1.66 (1H, m, CHCH₂H₆), 1.66 – 1.55 (1H, m, CHCH₂H₆), 1.51 – 1.44 (2H, m, CH₂CH₂NH), 1.43 (9H, s, 3 x CH₃), 1.42 – 1.31 (2H, m, CHCH₂CH₂); 13C NMR δ (126 MHz, MeOD) 177.16 (C), 158.56 (C), 79.82 (C), 55.02 (CH), 52.39 (CH₃), 41.07 (CH₂), 35.30 (CH₂), 30.70 (CH₂), 28.79 (3 x CH₃), 23.76 (CH₂); m/z (ESI+, MeCN) 261 ([M+H]⁺, 50%).

3-(3-Methyl-3H-diazirin-3-yl)propanoic acid 63

A solution of levulinic acid (500 mg, 4.31 mmol) in methanol (1 cm³) was cooled in an ice bath under an argon atmosphere. To this solution was added 7 N ammonia (5 cm³ in methanol) dropwise. The resulting solution was stirred for 3 h at 0 °C. A solution of hydroxylamine-O-sulfonic acid (584 mg, 5.17 mmol) in methanol (1 cm³) was added dropwise slowly to the reaction mixture, which was stirred overnight (16 h) and allowed to warm to rt. Nitrogen was bubbled through the solution for 1 h to remove the excess ammonia. The suspension was filtered and the filtrate was concentrated by rotary evaporation. The yellow oil was dissolved in methanol (1 cm³) and stirred on ice for 5 min in the dark. Triethylamine (662 mg, 6.55 mmol) was added dropwise slowly and allowed to stir for 5 min. Iodine (1.88 g, 7.97 mmol) was added
slowly until the brown-red colour persisted for longer than 5 min. The reaction solution was diluted with ethyl acetate (10 cm$^3$) and washed with HCl (1 x 10 cm$^3$; 1N aq.) and sodium thiosulfate (2 x 10 cm$^3$; 10% aq) until the organic layer was colourless. The aqueous layer was further extracted with ethyl acetate (2 x 10 cm$^3$), the organic layers were combined, washed with brine (20 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo to afford the product 63 as an orange-brown residue (330 mg, 60%). IR (neat, cm$^{-1}$) 3000-2900 (OH), 1710 (CO), 1585 (N=N); $^1$H NMR $\delta$ (601 MHz, CDCl$_3$) 2.26 – 2.22 (2H, m, CH$_2$CO), 1.78 – 1.66 (2H, m, CH$_2$CH$_2$CO), 1.04 (3H, s, C(CH$_3$)$_3$); $^{13}$C NMR $\delta$ (151 MHz, CDCl$_3$) 178.30 (C), 29.50 (CH$_2$), 28.57 (CH$_2$), 25.17 (C), 19.82 (CH$_3$); $m/z$ (ESI−, MeCN) 380 (100%), 126 ([M−H]$^-$, 50).

**Methyl (2S)-2-[(4-benzoylephenyl)formamido]-6-[(tert-butoxy)carbonyl]amino]hexanoate 64**

![Chemical Structure](image)

To H-Lys(Boc)-OMe 60 (265 mg, 1.02 mmol) in DMF (20 cm$^3$) was added 4-benzoyle benzoic acid 61 (298 mg, 1.06 mmol), EDC•HCl (224 mg, 1.17 mmol), HOBT (158 mg, 1.17 mmol), and NEt$_3$ (118 mg, 1.17 mmol), and the reaction was stirred for 48 h at rt under darkness. The solvent was then removed in vacuo and the residue was resuspended in DCM (50 cm$^3$), and washed with H$_2$O (50 cm$^3$) and brine (50 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo. The solid was purified by column chromatography (EtOAc:hexane, 1:1) to give the desired compound as a colourless foam (446 mg, 93.6%). R$_f$ (EtOAc:hexane, 1:1) = 0.34; IR (neat, cm$^{-1}$) 3500-3200 (NH), 1741 (CO), 1691 (CO), 1651 (CO); $^1$H NMR $\delta$ (500 MHz, CDCl$_3$) 7.94 (2H, d, $J = 8.3$ Hz, 2 x ArH), 7.86 (2H, d, $J = 8.3$ Hz, 2 x ArH), 7.80 (2H, dd, $J = 8.3$, 1.3 Hz, 2 x ArH), 7.62 (1H, t, $J = 7.4$ Hz, ArH), 7.50 (2H, t, $J = 7.4$ Hz, 2 x ArH), 6.87 (1H, br d, $J = 6.4$ Hz, NH), 4.82 (1H, dd, $J = 12.4$, 7.3 Hz, NHCH), 4.59 (1H, br s, NH/Boc),
3.80 (3H, s, OCH₃), 3.19 – 3.08 (2H, m, CH₂NH), 2.04 – 1.97 (1H, m, CHCH₃H₆), 1.90 – 1.80 (1H, m, CHCH₃H₆), 1.60 – 1.37 (4H, m, 2 x CH₂), 1.40 (9H, br s, C(CH₃)₃); 

1³C NMR δ (126 MHz, CDCl₃) 196.04 (C), 173.03 (C), 166.51 (C), 156.30 (C), 140.52 (C), 137.26 (C), 137.20 (C), 133.04 (CH), 130.28 (2 x CH), 130.24 (2 x CH), 128.60 (2 x CH), 127.31 (2 x CH), 79.33 (C), 52.77 (CH), 52.75 (CH₃), 40.14 (CH₂), 32.25 (CH₂), 29.87 (CH₂), 28.53 (3 x CH₃), 22.62 (CH₂); m/z (ESI+, MeCN) 491 ([M+Na]⁺, 15%), 413 ([M-Boc+2Na]⁺, 7), 369 ([M-Boc+H]⁺, 100); HRMS (ESI+, MeCN) [M+Na]⁺ found 491.2151, C₂₆H₂₂N₂O₆Na requires 491.2153.

Methyl (2S)-6-azido-2-[(4-benzoylphenyl)formamido]hexanoate 65

To a solution of benzophenone-Lys(Boc)-OMe 64 (446 mg, 0.953 mmol) in DCM (10 cm³) was added TFA (8.00 cm³, 0.104 mol), and the reaction was stirred for 2 h at rt under N₂. The solvent was removed in vacuo, and the residue was dried under high vacuum (2 h) to remove residual TFA. The residue was resuspended in methanol (10 cm³), to this was added potassium carbonate (569 mg, 4.12 mmol), CuSO₄•5H₂O (9.5 mg, 0.038 mmol), and imidazole-1-sulfonyl azide (240 mg, 1.14 mmol), and the reaction was stirred for 4 h at rt in the dark under N₂. The solvent was removed in vacuo, and the residue was resuspended with EtOAc (100 cm³), and was washed with HCl (2 x 50 cm³; 2 N aq), and brine (50 cm³), dried (MgSO₄) and concentrated in vacuo. The solid was purified by column chromatography (EtOAc:hexane, 1:1) to give the desired compound as a colourless solid (318 mg, 85%). Rf (EtOAc:hexane, 1:1) = 0.5; IR (neat, cm⁻¹) 3500-3200 (NH), 2096 (N₃), 1741 (CO), 1651 (CO); ¹H NMR δ (500 MHz, CDCl₃) 7.92 (2H, d, J = 8.5 Hz, 2 x ArH), 7.87 (2H, d, J = 8.5 Hz, 2 x ArH), 7.80 (1H, dd, J = 8.3, 1.3 Hz, 2 x ArH), 7.62 (1H, br t, J = 7.4 Hz, ArH), 7.50 (1H, br t, J = 7.7 Hz, 2 x ArH), 6.77 (1H, br d, J = 7.6 Hz, NH), 4.87 (1H, td, J = 7.3, 5.4 Hz, NHCH₃), 3.82 (3H, s, OCH₃), 3.30 (2H, m, CH₂N₃), 2.10 – 2.00 (1H, m,
Experimental

CHCH₂H₆), 1.86 (1H, dddd, J = 13.8, 10.2, 7.0, 5.4 Hz, CHCH₄H₆), 1.72 – 1.60 (2H, m, CH₂CH₂N₃), 1.54 – 1.40 (2H, m, CH₂CH₂CH₂); ¹³C NMR δ (126 MHz, CDCl₃) 196.00 (C), 172.95 (C), 166.33 (C), 140.61 (C), 137.18 (C), 137.17 (C), 133.07 (CH), 130.33 (2 x CH), 130.24 (2 x CH), 128.62 (2 x CH), 127.21 (2 x CH), 52.86 (CH), 52.62 (CH₃), 51.23 (CH₂), 32.36 (CH₂), 28.59 (CH₂), 22.63 (CH₂); m/z (ESI–, MeCN) 393 ([M–H]⁻, 100%); HRMS (ESI, MeCN) [M–H]⁻ found 393.1552, C₂₁H₂₁N₄O₄ requires 393.1557.

(2S)-6-Azido-2-[(4-benzoylphenyl)formamido]hexanoic acid 66

To benzophenone-Lys(N₃)-OMe 65 (319 mg, 0.808 mmol) in THF (10 cm³) was added sodium hydroxide (7 cm³, 2 N aq), and the reaction mixture was stirred at rt for 1.5 h under an inert atmosphere. The solvent was then removed in vacuo and the residue was purified by flash chromatography (DCM:MeOH:AcOH, 18:1:1) to give the product 66 as a colourless oil (292 mg, 95%). Rf (DCM:MeOH:AcOH, 18:1:1) = 0.41; IR (neat, cm⁻¹) 3300-2870 (NH/OH), 2096 (N₃), 1647 (CO); ¹H NMR δ (500 MHz, CDCl₃) 7.90 (2H, d, J = 8.5 Hz, 2 x ArH), 7.81 (2H, d, J = 8.5 Hz, 2 x ArH), 7.77 (2H, dd, J = 8.3, 1.3 Hz, 2 x ArH), 7.61 (1H, tt, J = 7.0, 1.3 Hz, ArH), 7.48 (2H, t, J = 7.7 Hz, 2 x ArH), 7.06 (1H, d, J = 7.7 Hz, NH), 4.85 (1H, td, J = 7.5, 5.4 Hz, NHCH), 3.34 – 3.24 (2H, m, CH₂N₃), 2.10 – 2.04 (1H, m, CHCH₄H₆), 1.95 – 1.86 (1H, m, CHCH₄H₆), 1.72 – 1.47 (4H, m, CH₂CH₂N₃ + CHCH₂CH₂); ¹³C NMR δ (126 MHz, CDCl₃) 196.18 (C), 175.97 (C), 167.14 (C), 140.67 (C), 136.98 (C), 136.84 (C), 133.18 (CH), 130.28 (2 x CH), 130.25 (2 x CH), 128.63 (2 x CH), 127.33 (2 x CH), 52.78 (CH), 51.20 (CH₂), 31.77 (CH₂), 28.52 (CH₂), 22.75 (CH₂); m/z (ESI+, MeCN) 403 ([M+Na]⁺, 40%), 381 ([M+H]⁺, 100%); HRMS (ESI+, MeCN) (ESI+, MeCN) [M+H]⁺ found 381.1561, C₂₀H₂₁N₄O₄ requires 381.1557.
2,5-Dioxopyrrolidin-1-y1 3-(3-methyl-3H-diazirin-3-yl)propanoate

To a stirred solution of diazirine acid 63 (394 mg, 3.07 mmol) and N-hydroxysuccinimide (389 mg, 3.38 mmol) in DCM (5 cm³), was added dropwise N,N'-dicyclohexylcarbodiimide (697 mg, 3.38 mmol) in anhydrous DCM (5 cm³). The reaction was stirred overnight (18 h) at rt in the dark. The reaction was filtered to remove the precipitated DCU, and the filtrate was concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 99:1) to give the product as a colourless solid (456 mg, 68%). \( R_f (\text{DCM:MeOH, 99:1}) = 0.31; \text{IR} \) (neat, cm\(^{-1}\)) 1815 (CO), 1784 (CO), 1734 (CO), 1587 (N=N diazirine); \(^1\text{H NMR} \) \( \delta \) (500 MHz, CDCl\(_3\)) 2.84 (4H, br s, 2 x CH\(_2\)CON), 2.52 (2H, dd, \( J = 8.3, 7.4 \text{ Hz, CH}_2\)CO\(_2\)), 1.85 – 1.77 (2H, m, CH\(_2\)CH\(_2\)CO\(_2\)), 1.08 (3H, s, CCH\(_3\)); \(^{13}\text{C NMR} \) \( \delta \) (126 MHz, CDCl\(_3\)) 169.02 (2 x C), 167.73 (C), 29.68 (CH\(_2\)), 25.88 (CH\(_2\)), 25.72 (2 x CH\(_2\)), 24.85 (C), 19.63 (CH\(_3\)); \( m/z \) (ESI+, MeCN) 247 ([M+Na]\(^+\), 100%), 225 ([M+H]\(^+\), 80).

Methyl (2S)-6-azido-2-[3-(3-methyl-3H-diazirin-3-yl)propanamido]hexanoate 68

To a stirred solution of diazirine-NHS (210 mg, 0.932 mmol) in DMF (10 cm³) was added H-Lys(N\(_3\))-OMe (173 mg, 0.932 mmol) and triethylamine (472 mg, 4.66 mmol). The reaction mixture was stirred overnight (18 h) in the dark at rt under an inert atmosphere. The reaction was quenched with the addition of \( \text{H}_2\text{O} \) (15 cm³) and NaHCO\(_3\) (15 cm³; sat aq), and extracted with DCM (3 x 50 cm³). The combined organic layer was dried (MgSO\(_4\)) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 19:1) to give the desired product 68 as a
Experimental

colourless oil (151 mg, 55%).  $R_f$ (DCM:MeOH, 9:1) = 0.6;  $\text{IR}$ (neat, cm$^{-1}$) 3294 (NH), 2096 (N$_3$), 1743 (CO), 1651 (CO); $^1$H NMR $\delta$ (500 MHz, CDCl$_3$) 6.00 (1H, d, $J = 7.5$ Hz, NH), 4.62 (1H, td, $J = 7.5, 5.4$ Hz, CH$_2$NH), 3.75 (3H, s, OCH$_3$), 3.27 (2H, td, $J = 6.7, 1.6$ Hz, CH$_2$N$_3$), 2.12 – 1.98 (2H, m, CH$_2$CONH), 1.87 (1H, m, CHCH$_2$H$_b$), 1.82 – 1.73 (2H, m, CH$_2$CH$_2$CONH), 1.73 – 1.54 (4H, m, CHCH$_2$H$_b$ + CH$_2$CH$_2$N$_3$), 1.49 – 1.31 (2H, m, CH$_2$CH$_2$CH$_2$N$_3$), 1.02 (3H, s, CCH$_3$); $^{13}$C NMR $\delta$ (126 MHz, CDCl$_3$) 172.90 (C), 171.12 (C), 52.63 (CH$_3$), 51.99 (CH), 51.19 (CH$_2$), 32.16 (CH$_2$), 30.62 (CH$_2$), 29.92 (CH$_2$), 28.50 (CH$_2$), 25.46 (C), 22.49 (CH$_2$), 20.01 (CH$_3$); m/z (ESI+, MeCN) 319 ([M+Na]$^+$, 100%); HRMS (ESI+, MeCN) [M+Na]$^+$ found 319.1482, C$_{12}$H$_{20}$N$_6$O$_3$Na requires 319.1489.

Methyl-(2S)-6-[(tert-butoxycarbonyl)amino]-2-[3-(3-methyl-3H-diazirin-3-yl)propanamido]hexanoate 67

To H-Lys(Boc)-OMe (250 mg, 0.960 mmol) in DMF (10 cm$^3$) was added diazirine-acid 63 (135 mg, 1.06 mmol), EDC•HCl (212 mg, 1.10 mmol), HOBt (149 mg, 1.10 mmol), and NEt$_3$ (112 mg, 1.10 mmol), and the reaction was stirred for 48 h at rt under darkness. The solvent was then removed in vacuo and the residue was resuspended in DCM (50 cm$^3$), and washed with H$_2$O (50 cm$^3$) and brine (50 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo. The solid was purified by column chromatography (EtOAc:hexane, 1:1) to give the desired compound as a colourless solid (301 mg, 84%).  $R_r$ (EtOAc:hexane, 1:1) = 0.22; mp pending; IR (neat, cm$^{-1}$) 3500-3200 (NH), 1718 (CO), 1689 (CO), 1654 (CO), 1523 (N=N, diazirine); $^1$H NMR $\delta$ (500 MHz, CDCl$_3$) 6.10 (1H, br d, $J = 5.3$ Hz, CONH), 4.57 (2H, br dd, $J = 12.3, 7.3$ Hz, NHBoc + NHCH), 3.74 (3H, s, OCH$_3$), 3.10 (2H, br q, $J = 6.2$ Hz, CH$_2$NH), 2.12 – 1.99 (2H, m, CH$_2$CONH), 1.91 – 1.79 (1H, m, CHCH$_2$H$_b$), 1.76 (2H, m, CH$_2$CH$_2$CONH), 1.74 – 1.65 (1H, m, CHCH$_3$H$_b$), 1.53 – 1.45 (2H, m, CH$_2$CH$_2$NH), 1.44 (9H, s, C(CH$_3$)$_3$),
1.42 – 1.28 (2H, m, CHCH\(_2\)CH\(_2\)), 1.03 (3H, s, CCH\(_3\)); \(^{13}\)C NMR \(\delta\) (126 MHz, CDCl\(_3\)) 173.00 (C), 171.29 (C), 156.32 (C), 79.35 (C), 52.55 (CH), 52.15 (CH\(_3\)), 40.06 (CH\(_2\)), 32.02 (CH\(_2\)), 30.61 (CH\(_2\)), 29.95 (CH\(_2\)), 29.77 (CH\(_2\)), 28.57 (3 x CH\(_2\)), 25.50 (C), 22.42 (CH\(_2\)), 20.03 (CH\(_3\)); \(m/z\) (ESI+, MeCN) 393 ([M+Na]\(^+\), 40%), 271 ([M-Boc+H]\(^+\), 100); HRMS (ESI+, MeCN) [M+Na]\(^+\) found 393.2112, C\(_{17}\)H\(_{30}\)N\(_4\)O\(_5\)Na requires 393.2108.

**Experimental**

(2S)-6-Azido-2-[3-(methyl-3H-diazirin-3-yl)propanamido]hexanoic acid 69

To a solution of diazirine-Lys(Boc)-OMe 67 (301 mg, 0.812 mmol) in THF (15 cm\(^3\)) was added NaOH (7 cm\(^3\); 2 N aq) and the reaction was stirred for 2 h at rt under darkness. The solvent was removed in vacuo, and the residue was resuspended in ether. The organic layer was washed with HCl (2 x 25 cm\(^3\); 1 N aq), brine (25 cm\(^3\), sat aq), dried (MgSO\(_4\)) and concentrated in vacuo to give a yellow oil which was used directly in the next step. To crude diazirine-Lys(Boc)-OH (289 mg, 0.810 mmol) in DCM (10 cm\(^3\)) was added TFA (2.07 cm\(^3\); 12.2 mmol), and the reaction was stirred for 2 h at rt. The solvent was removed in vacuo, and the residue was dried under high vacuum (2 h) to remove residual TFA. The residue was resuspended in methanol (10 cm\(^3\)). To this solution was added potassium carbonate (484 mg, 3.50 mmol), CuSO\(_4\)•5H\(_2\)O (8.10 mg, 0.0324 mmol), and imidazole-1-sulfonyl azide (204 mg, 0.973 mmol), and the reaction was stirred for 4 h at rt under darkness. The solvent was removed in vacuo, and the residue was resuspended in EtOAc (100 cm\(^3\)), which was washed with HCl (2 x 50 cm\(^3\); 2 N aq), and brine (50 cm\(^3\)), dried (MgSO\(_4\)) and concentrated in vacuo. The residue was then resuspended in ether (50 cm\(^3\)), washed with NaOH (50 cm\(^3\); 1 N aq), the organic layer was disposed and the aqueous layer was acidified with HCl (50 cm\(^3\); 1 N aq). The aqueous layer was extracted with ether (2 x 50 cm\(^3\)). The combined ether extracts were washed with NaHCO\(_3\) (20 cm\(^3\); sat aq) and brine (20 cm\(^3\)), dried (MgSO\(_4\)) and concentrated in vacuo to give the desired
product 69 as a yellow oil (142 mg, 62%). \( \text{Rf} \) (DCM:MeOH, 9:1) = 0.1; \( \text{IR} \) (neat, cm\(^{-1}\)) 3400-3200 (NH, OH), 2094 (N\(_3\)), 1732 (CO), 1645 (CO), 1537 (N=N, diazirine); \(^1\)\text{H NMR} \( \delta \) (500 MHz, CDCl\(_3\)) 6.03 (1H, d, \( J = 7.5 \text{ Hz} \), NH), 4.62 (1H, dd, \( J = 12.9, 7.5 \text{ Hz} \), NHCH), 3.30 (2H, t, \( J = 6.7 \text{ Hz} \), CH\(_2\)N\(_3\)), 2.14 – 2.01 (2H, m, CH\(_2\)CONH), 2.00 – 1.91 (1H, m, CHCH\(_2\)N\(_3\)), 1.84 – 1.72 (3H, m, CHCH\(_2\)H\(_3\) + CH\(_2\)CH\(_2\)CONH), 1.69 – 1.58 (2H, m, CH\(_2\)CH\(_2\)N\(_3\)), 1.54 – 1.41 (2H, m, CH\(_2\)CH\(_2\)CH\(_2\)N\(_3\)), 1.04 (3H, s, CH\(_3\)); \(^{13}\)\text{C NMR} \( \delta \) (126 MHz, CDCl\(_3\)) 175.32 (C), 172.01 (C), 52.12 (CH), 51.21 (CH\(_2\)), 31.67 (CH\(_2\)), 30.62 (CH\(_2\)), 29.93 (CH\(_2\)), 28.51 (CH\(_2\)), 25.45 (C), 22.57 (CH\(_3\)), 20.03 (CH\(_3\)); \( m/z \) (ESI\(^{-}\), MeCN) 563 ([2M–H]\(^{-}\), 100%); 281 ([M–H]\(^{-}\), 30), HRMS (ESI\(^{-}\), MeCN) [M–H]\(^{-}\) found 281.1364, C\(_{11}\)H\(_{17}\)N\(_6\)O\(_3\) requires 281.1357.

\( \text{N-(3-\{4-[(E)-2-\{4-[2-\{5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido\}ethyl]carbamoyl]phenyl]diazen-1-yl]phenoxy}propyl)-6-azido-2-\{3-(methyl-3H-diazirin-3-yl)propanamido\}hexanamide 70\)

To a solution of biotin linker amide 54 (17.8 mg, 0.0314 mmol) in DMF (1 cm\(^3\)), was added HOBt (5.3 mg, 0.0389 mmol), EDC (7.5 mg, 0.0392 mmol) and DIPEA (11 \( \mu \)L, 0.0627 mmol). The solution was stirred for 5 min, after which diazirine-Lys(N\(_3\))-OH 69 (9.7 mg, 0.0345 mmol) in DMF (0.5 cm\(^3\)) was added and the reaction was stirred for 48 h at rt in the dark. The solvent was then removed \textit{in vacuo}, and the residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 70 as an orange solid (6.8 mg, 26%). \( \text{Rf} \) (DCM:MeOH, 9:1) = 0.4; \( \text{IR} \) (neat, cm\(^{-1}\)) 3400-3200 (NH), 2098 (N\(_3\)), 1697 (CO), 1635 (CO), 1546 (N=N); \(^1\)\text{H NMR} \( \delta \) (500 MHz, (CD\(_3\))\(_2\)SO) 8.64 (1H, t, \( J = 5.5 \text{ Hz} \), NH), 8.06 – 7.99 (4H, m, 2 x ArH + 2 x NH), 7.95
(1H, m, NH), 7.92 (2H, d, J = 8.9 Hz, 2 x ArH), 7.89 (2H, d, J = 8.5 Hz, 2 x ArH), 7.13 (2H, d, J = 8.9 Hz, 2 x ArH), 6.40 (1H, s, NH), 6.33 (1H, s, NH), 4.28 (1H, dd, J = 7.7, 5.1 Hz, NHCHCH₂), 4.20 (1H, td, J = 8.4, 5.5 Hz, COCHNH), 4.14 – 4.05 (3H, m, NHCHCH + OCH₂), 3.38 – 3.31 (2H, m, CH₂NH), 3.30 – 3.20 (6H, m, 2 x CH₂NH + CH₂N₃), 3.04 (1H, ddd, J = 8.5, 6.2, 4.2 Hz, NHCHCH₃), 2.79 (1H, dd, J = 12.4, 5.1 Hz, CH₃H₃S), 2.56 (1H, d, J = 12.4 Hz, CH₂H₃S), 2.11 – 2.00 (4H, m, 2 x CH₂CONH), 1.89 (2H, p, J = 6.7 Hz, OCH₂CH₂), 1.67 – 1.56 (2H, m, SCHCH₂H₃ + COCHCH₃H₃), 1.55 (2H, t, J = 7.7 Hz, CH₂CCH₃), 1.54 – 1.40 (4H, m, SCHCH₃H₃ + COCHCH₃H₃ + CH₂CH₂N₃), 1.37 – 1.21 (4H, m, SCHCH₂CH₂ + COCHCH₂CH₂), 0.97 (3H, s, CCH₃); ¹³C NMR δ (126 MHz, (CD₃)₂SO) 172.38 (C), 171.57 (C), 170.72 (C), 165.59 (C), 162.65 (C), 161.76 (C), 153.38 (C), 146.14 (C), 135.96 (C), 128.43 (2 x CH), 124.82 (2 x CH), 121.96 (2 x CH), 115.12 (2 x CH), 65.73 (CH₂), 60.96 (CH), 59.16 (CH), 55.35 (CH), 52.34 (CH), 50.50 (CH₂), 39.80 (CH₂), 39.39 (CH₂), 38.15 (CH₂), 35.32 (CH₂), 35.24 (CH₂), 31.55 (CH₂), 29.80 (CH₂), 29.56 (CH₂), 28.67 (CH₂), 28.14 (CH₂), 28.01 (CH₂), 27.87 (CH₂), 25.77 (C), 25.22 (CH₂), 22.56 (CH₂), 19.24 (CH₃); m/z (ESI+, MeCN) 854 ([M+Na]⁺, 75%), 832 ([M+H]⁺, 100); HRMS (ESI+, MeCN) (ESI+, MeCN) [M+H]⁺ found 832.4034, C₃₉H₅₄N₁₃O₆S requires 832.4035.


To a solution of biotin linker amide 54 (25.6 mg, 0.0451 mmol) in DMF (1 cm³), was added HOBt (7.0 mg, 0.0518 mmol), EDC (9.9 mg, 0.0518 mmol) and DIPEA (9 µL, 0.0518 mmol). The solution was stirred for 5 min, after which benzophenone-Lys(N₃)-
OH 66 (18.9 mg, 0.0496 mmol) in DMF (0.5 cm³) was added and the reaction was stirred for 48 h at rt in the dark. The solvent was then removed in vacuo, and the residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 71 as an orange solid (12.5 mg, 30%). \( ^1H \text{ NMR} \ \delta \) (500 MHz, (CD₃)₂SO) 8.67 (1H, d, \( J = 7.9 \) Hz, NH), 8.64 (1H, t, \( J = 5.5 \) Hz, NH), 8.16 (1H, t, \( J = 5.5 \) Hz, NH), 8.06 (2H, d, \( J = 8.2 \) Hz, 2 x ArH), 8.02 (2H, d, \( J = 8.5 \) Hz, 2 x ArH), 7.95 (1H, t, \( J = 5.6 \) Hz, NH), 7.89 (2H, d, \( J = 9.0 \) Hz, 2 x ArH), 7.87 (2H, d, \( J = 8.5 \) Hz, 2 x ArH), 7.80 (2H, d, \( J = 8.2 \) Hz, 2 x ArH), 7.78 – 7.71 (2H, m, 2 x ArH), 7.69 (1H, t, \( J = 7.5 \) Hz, ArH), 7.58 (2H, q, \( J = 7.6 \) Hz, 2 x ArH), 7.12 (2H, d, \( J = 9.0 \) Hz, 2 x ArH), 6.40 (1H, s, NH), 6.33 (1H, s, NH), 4.43 (1H, td, \( J = 8.6 \), 5.7 Hz, COCHNH), 4.27 (1H, dd, \( J = 7.6 \), 5.2 Hz, NHCHOH), 4.13 (2H, t, \( J = 6.3 \) Hz, OCH₂), 4.09 (1H, ddd, \( J = 7.3 \), 4.4, 1.7 Hz, NHCHOH), 3.38 – 3.19 (8H, m, 3 x CH₂NH + CH₂N₃), 3.04 (1H, ddd, \( J = 8.2 \), 6.2, 4.4 Hz, NHCHOH), 2.79 (1H, dd, \( J = 12.5 \), 5.0 Hz, CH₃H₂S), 2.56 (1H, d, \( J = 12.5 \) Hz, CH₃H₂S), 2.07 (2H, dt, \( J = 13.6 \), 7.4 Hz, CH₂CONH), 1.99 – 1.88 (2H, m, OCH₂CH₂), 1.86 – 1.68 (2H, m, COCHCH₃H₆ + COCHCH₃H₆), 1.65 – 1.21 (10H, m, SCHCH₃H₆ + SCHCH₃H₆ + SCHCH₃CH₂ + CH₂CH₂CO + COCHCH₂CH₂ + CH₂CH₂N₃); \( ^13 \text{C NMR} \ \delta \) (126 MHz, (CD₃)₂SO) 195.40 (C), 172.38 (C), 171.58 (C), 165.65 (C), 165.59 (C), 162.65 (C), 161.76 (C), 153.37 (C), 146.12 (C), 139.19 (C), 137.41 (C), 136.65 (C), 135.94 (C), 132.99 (CH) 129.66 (2 x CH), 129.30 (2 x CH), 128.64 (2 x CH), 128.41 (2 x CH), 127.74 (2 x CH), 124.80 (2 x CH), 121.95 (2 x CH), 115.11 (2 x CH), 65.77 (CH₂), 60.96 (CH), 59.15 (CH), 55.35 (CH), 53.55 (CH), 50.50 (CH₂), 39.81 (CH₂), 39.39 (CH₂), 38.15 (CH₂), 35.44 (CH₂), 35.24 (CH₂), 31.07 (CH₂), 28.69 (CH₂), 28.14 (CH₂), 28.01 (CH₂), 27.90 (CH₂), 25.22 (CH₂), 22.98 (CH₂); \textit{m/z} (ESI+, MeCN) 952 ([M+Na]⁺, 100%), 930 ([M+H]⁺, 80); HRMS (ESI+, MeCN) (ESI+, MeCN) [M+Na]⁺ found 952.3896, C₄₈H₅₃N₁₁O₇Sn requires 952.3899.

3-Azidopropan-1-amine 74

![3-Azidopropan-1-amine](image)

To a stirred solution of 3-chloropropylamine hydrochloride (561 mg, 4.31 mmol) in H₂O (5 cm³), was added sodium azide (839 mg, 12.9 mmol) and the mixture was
heated to 80 °C. After 15 h, potassium hydroxide pellets were added to basify the solution, followed by extraction with diethyl ether (3 x 20 cm³). The combined organic phases were dried (MgSO₄) and concentrated in vacuo to give the desired product 74 as a colourless volatile oil (421 mg, 98% yield); \textbf{IR} (neat, cm⁻¹) 3400 (NH₂), 2096 (N₃); \textbf{¹H NMR} δ (400 MHz, CDCl₃) 3.25 (2H, t, $J = 6.8$ Hz, CH₂NH₂), 2.67 (2H, t, $J = 6.8$ Hz, CH₂CH₂CH₂), 1.28 (2H, br s, NH₂); \textbf{¹³C NMR} δ (126 MHz, CDCl₃) 48.86 (CH₂), 39.00 (CH₂), 32.11 (CH₂).

All spectroscopic data were in good agreement with the literature.²¹⁸

5-[(3S,4S,6R)-2-Oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-(3-azidopropyl)-pentanamide 75

D-biotin (100 mg, 0.41 mmol) was dissolved in DMF (10 cm³). HOBt (108 mg, 0.80 mmol) and DIC (124 µL, 0.80 mmol) were added to the solution and stirred for 10 min at rt. 3-azido-1-propyamine 74 (200 mg, 1.99 mmol) was added to the solution and the reaction was heated to 60 °C by microwave irradiation for 20 min. The organic solvent was removed in vacuo and the crude material was purified by flash chromatography (2 – 10% MeOH in DCM) to give the product 75 as a colourless solid (90.9 mg, 67% yield). \textbf{Rf} (DCM:MeOH, 9:1 = 0.2); \textbf{IR} (neat, cm⁻¹) 3400-3150 (NH), 2096 (N₃), 1700 (CO), 1645 (CO); \textbf{¹H NMR} (500 MHz, CD₃OD) δ 4.49 (1H, dd, $J = 7.9$, 5.0 Hz, CHCH₂S), 4.30 (1H, dd, $J = 7.9$, 4.5 Hz, CHCHS), 3.35 (2H, t, $J = 6.7$ Hz, CH₂N₃), 3.27 – 3.23 (2H, m, CH₂NH), 3.23–3.18 (1H, m, CHS), 2.93 (1H, dd, $J = 12.8$, 5.0 Hz, CH₂H₂S), 2.71 (1H, d, $J = 12.7$ Hz, CH₂H₂S), 2.21 (2H, t, $J = 7.2$ Hz, CH₂CO), 1.81–1.72 (2H, m, CH₂CH₂N₃), 1.73–1.53 (4H, m, SCHCH₂ + CH₂CH₂CO), 1.49–1.40 (2H, m, CH₂CH₂CH₂CO); \textbf{¹³C NMR} δ (126 MHz, CD₃OD) 176.17 (C), 166.13 (C), 166.13 (C), 63.40 (CH), 61.64 (CH), 57.00 (CH), 50.10 (CH₂), 41.02 (CH₂), 37.70 (CH₂), 36.78 (CH₂), 29.79 (CH₂), 29.75 (CH₂), 29.49 (CH₂), 26.85 (CH₂); \textbf{m/z} (ESI⁺,
MeCN) 349 ([M+Na]^+, 50%), 327 ([M+H]^+, 100); **HRMS** (ESI+, MeCN) (ESI+, MeCN) [M+H]^+ found 327.1596, C_{13}H_{23}N_{6}O_{2}S requires 327.1598.

All spectroscopic data were in good agreement with the literature.\(^{219}\)

**5-[(3aS,4S,6aR)-2-Oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-(2-aminoethyl)pentanamide 76**

To a stirred solution of diaminoethane (30.1 mg, 0.501 mmol) and triethylamine (249 mg, 2.45 mmol) in DMF (5 cm\(^3\)), was added biotin-NHS 52 (171 mg, 0.501 mmol) in DMF (2 cm\(^3\)) dropwise. The reaction was stirred overnight (18 h) at room temperature under an inert atmosphere. The precipitate formed was filtered off, and the filtrate was concentrated in vacuo. The residue was precipitated into excess cold hexane (100 cm\(^3\)). The white precipitate formed was filtered and washed with isopropanol (3 x 5 cm\(^3\)) to give a white powder which was dried overnight (18 h) in vacuo. This gave the desired product 76 as a white solid (129 mg, 90%). **R\(_f\)** (DCM:MeOH, 4:1) = 0.2; **IR** (neat, cm\(^{-1}\)) 3500-3000 (NH), 1668 (CO), 1651 (CO); **\(^1\)H NMR** \(\delta\) (500 MHz, (CD\(_3\))\(_2\)SO) 7.75 (1H, t, \(J = 5.3\) Hz, CONH), 6.41 (1H, s, NHCHCH), 6.34 (1H, s, NHCHCH\(_2\)), 4.32 – 4.27 (2H, m, NHCHCH\(_2\)), 4.16 – 4.10 (2H, m, NHCHCH), 3.13 – 3.07 (1H, m, NHCHCH\(_2\)), 3.03 (2H, dd, \(J = 12.2, 6.3\) Hz, NHCH\(_2\)), 2.82 (1H, dd, \(J = 12.4, 5.1\) Hz, CH\(_3\)H\(_9\)S), 2.58 – 2.53 (3H, m, CH\(_3\)H\(_6\)S + CH\(_2\)NH\(_2\)), 2.06 (2H, t, \(J = 7.4\) Hz, CH\(_2\)CO), 1.61 (1H, ddt, \(J = 12.3, 9.9, 6.1\) Hz, SCHCH\(_3\)H\(_9\)), 1.56 – 1.40 (3H, m, SCHCH\(_3\)H\(_9\) + CHCH\(_2\)CH\(_2\)), 1.38 – 1.24 (2H, m, CH\(_2\)CH\(_2\)CO); **\(^13\)C NMR** \(\delta\) (126 MHz, (CD\(_3\))\(_2\)SO) 172.05 (C), 162.68 (C), 61.02 (CH), 59.16 (CH), 55.39 (CH), 41.90 (CH\(_2\)), 41.24 (CH\(_2\)), 39.56 (CH\(_2\)), 35.19 (CH\(_2\)), 28.19 (CH\(_2\)), 28.01 (CH\(_2\)), 25.25 (CH\(_2\)); **m/z** (ESI+, MeCN) 287 ([M+H]^+, 100%), 309 ([M+Na]^+, 40); **HRMS** (ESI+, MeCN) [M+H]^+ found 287.1534, C\(_{12}\)H\(_{22}\)N\(_4\)O\(_2\)S requires 287.1536.

All spectroscopic data were in good agreement with the literature.\(^{220}\)
(2S)-N-(2-{5-[3aS,4S,6aR]-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido}ethyl)-6-azido-2-[3-(3-methyl-3H-diazirin-3-yl)propanamido]hexanamide 78

To a solution of 2-aminoethyl biotin amide 76 (50.0 mg, 0.175 mmol) in DMF (4 cm³), was added HOBt (49.3 mg, 0.216 mmol), EDC (41.8 mg, 0.216 mmol) and DIPEA (45.1 mg, 0.349 mmol). The solution was stirred for 5 min, after which diazirine-Lys(N₃)-OH 69 (49.3 mg, 0.175 mmol) in DMF (2 cm³) was added and the reaction was stirred for 48 h at rt in the dark. The solvent was then removed in vacuo, the residue solubilised in EtOAc (20 cm³) and subsequently washed with water (2 x 10 cm³) and brine (10 cm³, sat aq). The organic phase was dried (MgSO₄) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 78 as a colourless solid (30.8 mg, 31.9%).

Rf (DCM:MeOH, 9:1) = 0.11; IR (neat, cm⁻¹) 3500-3200 (NH), 2095 (N₃) 1734 (CO), 1653 (CO), 1560 (N=N, diazirine); ¹H NMR δ (500 MHz, CD₃OD) 4.50 (1H, dd, J = 7.9, 5.0 Hz, CH₂S), 4.32 (1H, dd, J = 7.9, 4.5 Hz, CHCHS), 4.22 (1H, dd, J = 9.1, 5.2 Hz, CHNH), 3.38 – 3.19 (7H, m, 2 x CH₂NH + CH₂N₃ + CHCHS), 2.94 (1H, dd, J = 12.7, 5.0 Hz, CH₂H₃S), 2.71 (1H, d, J = 12.7 Hz, CH₂H₂S), 2.26 – 2.14 (4H, m, 2 x CH₂CONH), 1.86 – 1.55 (8H, m, CH₂CH₂N₃ + SCHCH₂ + CH₂CH₂CONH + COCHCH₂), 1.68 (2H, t, J = 7.8 Hz, CH₂CCH₃), 1.55 – 1.27 (4H, m, CH₂CH₂CH₂N₃ + SCHCH₂CH₂), 1.02 (3H, s, CCH₃); ¹³C NMR δ (126 MHz, CD₃OD) 176.45 (C), 174.83 (C), 174.78 (C), 166.18 (C), 63.21 (CH), 61.70 (CH), 56.92 (CH), 54.95 (CH), 52.26 (CH₂), 41.02 (CH₂), 40.30 (CH₂), 40.00 (CH₂), 36.77 (CH₂), 32.48 (CH₂), 31.26 (CH₂), 31.05 (CH₂), 29.59 (CH₂), 29.49 (CH₂), 29.45 (CH₂), 26.77 (CH₂), 26.38 (C), 24.24 (CH₂), 19.76 (CH₃); m/z (ESI+, MeCN) 573 ([M+Na]⁺, 100%), 551 ([M+H]⁺, 20); HRMS (ESI+, MeCN) [M+H]⁺ found 551.2870, C₂₃H₉₀N₁₀O₄S requires 551.2871.
To a solution of 2-aminoethyl biotin amide 76 (50.0 mg, 0.175 mmol) in DMF (4 cm³), was added HOBt (49.3 mg, 0.349 mmol), EDC (41.8 mg, 0.216 mmol) and DIPEA (45.1 mg, 0.349 mmol). The solution was stirred for 5 min, after which benzophenone-Lys(N₃)-OH 66 (66.4 mg, 0.175 mmol) in DMF (2 cm³) was added and the reaction was stirred for 48 h at rt in the dark. The solvent was then removed in vacuo, the residue solubilised in EtOAc (20 cm³) and subsequently washed with water (2 x 10 cm³) and brine (10 cm³, sat aq). The organic phase was dried (MgSO₄) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 77 as a colourless solid (30.8 mg, 31.9%). **Rf** (DCM:MeOH, 9:1) = 0.23; **IR** (neat, cm⁻¹) 3500-3200 (NH), 2095 (N=O), 1730 (CO), 1651 (CO); **¹H NMR** δ (601 MHz, CD₂OD) 8.25 – 8.21 (1H, m, NH), 8.05 (2H, d, J = 8.5 Hz, 2 x ArH), 7.92 (1H, t, J = 5.1 Hz, NH), 7.86 (2H, d, J = 8.5 Hz, 2 x ArH), 7.81 (2H, dd, J = 8.3, 1.2 Hz, 2 x ArH), 7.67 (1H, t, J = 7.5 Hz, ArH), 7.55 (2H, t, J = 7.8 Hz, 2 x ArH), 4.51 – 4.44 (2H, m, NHCHCH₂ + CHNH), 4.27 (1H, dd, J = 7.9, 4.4 Hz, CHCHS), 3.56 – 3.48 (1H, m, CH₂H₂NH), 3.46 – 3.41 (1H, m, CH₂H₂NH), 3.35 – 3.32 (2H, m, CH₂N₃), 3.25 – 3.18 (2H, m, CH₂NH), 3.14 (1H, td, J = 7.3, 4.5 Hz, CHCHS), 2.88 (1H, dd, J = 12.7, 5.0 Hz, CH₂H₂S), 2.66 (1H, d, J = 12.7 Hz, CH₂H₂S), 2.18 (2H, dt, J = 28.9, 14.3, 7.2 Hz, CH₂CONH), 1.96 – 1.85 (2H, m, COCHCH₂), 1.72 – 1.54 (6H, m, CH₂CH₂N₃ + SCHCH₂ + CH₂H₂CO), 1.54 – 1.27 (4H, m, CH₂CH₂CH₂N₃ + SCHCH₂CH₂); **¹³C NMR** δ (151 MHz, CD₂OD) 197.74 (C), 176.58 (C), 174.95 (C), 169.48 (C), 166.18 (C), 141.58 (C), 138.58 (C), 138.39 (C), 134.17 (CH), 131.10 (2 x CH), 130.97 (2 x CH), 129.66 (2 x CH), 128.82 (2 x CH), 63.00 (CH), 61.73 (CH), 56.84 (CH), 55.91 (CH), 52.26 (CH₂), 40.97 (CH₂), 40.30 (2 x CH₂), 36.85 (CH₂), 32.43 (CH₂), 29.53 (CH₂), 29.44 (CH₂), 29.40 (CH₂), 26.83 (CH₂),
24.51 (CH₂); \textit{m/z} (ESI+, MeCN) 671 ([M+Na]+, 47%), 649 ([M+H]+, 100); \textbf{HRMS} (ESI+, MeCN) [M+H]+ found 649.2920, C_{32}H_{41}N_8O_5S requires 649.2915.
5.3 Experimental for Chapter 2

General procedure A: Cu(SO₄)-6H₂O click coupling

To a stirred solution of azide (~1.5 eq) in H₂O/tBuOH (1 cm³/0.015 mmol of azide) in a Wheaton vial at 25 °C, was added Cu(SO₄)-6H₂O (5 mol%) and sodium ascorbate (10 mol%). After 10 min, TBTA (5 mol%) was added. After 15 min, alkyne (~1 eq) was added, and the reaction was stirred overnight at 25 °C. The reaction mixture was then concentrated in vacuo.

General procedure B: Cu(MeCN)BF₄ click coupling

To a stirred solution of azide (1 eq) in DMF (1 cm³/0.015 mmol of azide) in a Wheaton vial at 25 °C, was added Cu(MeCN)BF₄ (5 mol%) and TBTA (5 mol%). After 15 min, alkyne (1 eq) was added, and the reaction was stirred overnight at 25 °C. The reaction mixture was then concentrated in vacuo, and purified by column chromatography.

(2R,3S,4S)-3-Acetoxy-4-hydroxy-2-(4'-methoxybenzyl)-1-propynyl-pyrrolidine 80a

To a stirred solution of anisomycin (20.2 mg, 0.076 mmol) in DMF (2 cm³), potassium carbonate (10.8 mg, 0.078 mmol) and propargyl bromide (9 µL, 0.08 mmol) were added. The reaction was stirred for 8 h at rt in the dark. The organic solvent was removed in vacuo and the crude material was purified by flash chromatography (5% MeOH in DCM) to give propargyl anisomycin 80a as a colourless oil (21 mg, 93% yield). Rₑ (5% MeOH in DCM) = 0.3; ¹H NMR δ (400 MHz, CDCl₃) 7.10 (2H, d, J = 8.6 Hz, ArH), 6.81 (2H, d, J = 8.6 Hz, ArH), 4.48 (1H, dd, J = 5.7, 1.7 Hz, CHOCOCH₃), 4.13 (1H, t, J = 8.1 Hz, CHO), 3.78 (3H, s, ArOCH₃), 3.60–3.55 (2H, m, HCCCH₂), 3.35 (1H, dd, J = 9.8, 7.1 Hz, NCH₃Hδ), 3.22 (1H, dt, J = 10.4, 5.2 Hz, NCH), 2.88–2.83 (1H, m, CH₂H₆Ar), 2.71 (1H, dd, J = 13.4, 10.3 Hz, CH₃H₆Ar),
2.65–2.58 (1H, m, NCH$_2$H$_2$), 2.30 (1H, t, $J = 2.4$ Hz, CHCCH$_2$), 2.14 (3H, s, OCOCH$_3$); $^{13}$C NMR $\delta$ (101 MHz, CDCl$_3$) 171.97 (C), 158.29 (C), 130.29 (C), 129.99 (2 x CH), 114.04 (2 x CH), 82.56 (CH), 76.71 (C), 75.80 (CH), 74.42 (CH), 63.66 (CH), 58.02 (CH$_2$), 55.38 (CH$_3$), 40.03 (CH$_2$), 32.15 (CH$_2$), 21.19 (CH$_3$); m/z (ESI+, MeCN) 304 ([M+H]$^+$, 100%); HRMS (ESI+, MeCN) [M+H]$^+$ found 304.1548, C$_{17}$H$_{22}$NO$_6$ requires 304.1543.

All spectroscopic data in good agreement with the literature.$^{140}$

(2R,3S,4S)-1-[(1-[(5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido)propyl]-1H-1,2,3-triazol-4-yl]methyl]-4-hydroxy-2-[(4-methoxyphenyl)methyl]pyrrolidin-3-yl acetate 89

**General procedure A** was followed: to a solution of biotin azide 75 (88.0 mg, 0.269 mmol) in H$_2$O/tBuOH (1:2, 3 cm$^3$), sodium ascorbate (11.5 mg, 0.0580 mmol) and copper sulphate (7 mg, 0.0280 mmol) were added. The reaction was stirred for 10 min, and then TBTA (10.7 mg, 0.0202 mmol) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (54 mg, 0.178 mmol) was added. The reaction was stirred overnight (18 h) at rt under an inert atmosphere. The organic layer was removed in vacuo, and the aqueous layer was extracted with DCM (3 x 10 cm$^3$) and EtOAc (3 x 10 cm$^3$). The combined organic layer was dried (MgSO$_4$) and concentrated in vacuo. Flash chromatography (2-10% MeOH in DCM) gave the desired product 89 as a colourless solid (64 mg, 59% yield).

**General procedure B** was followed: to a solution of biotin azide 75 (50 mg, 0.153 mmol) in DMF (2 cm$^3$), Cu(MeCN)$_4$BF$_4$ (2.4 mg, 5 mol%) was added. The reaction was stirred for 10 min, and then TBTA (4.1 mg, 5 mol%) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (46.5 mg, 0.153 mmol) was
added. The reaction was stirred overnight (18 h) at rt under an inert atmosphere. The organic layer was removed in vacuo, and the residue was purified by column chromatography (2-10% MeOH in DCM) to give the product 89 as a colourless solid (75 mg, 78%).

Rt (DCM:MeOH, 4:1) = 0.37; $^1$H NMR $\delta$ (400 MHz, CD$_3$OD) 7.95 (1H, s, NCHC, triazole), 7.12 (2H, d, $J = 8.6$ Hz, ArHf), 6.83 (2H, d, $J = 8.6$ Hz, ArHf), 4.64 (1H, dd, $J = 4.6$, 1.4 Hz, CHOCOCH$_3$), 4.51–4.47 (1H, m, CHCH$_2$S), 4.45 (2H, t, $J = 5.7$ Hz, CH$_2$NCHC), 4.29 (1H, dd, $J = 7.9$, 4.5 Hz, CHCHS), 4.04 (1H, d, $J = 14.2$ Hz, CCH$_2$H$_b$N), 4.00 (1H, dd, $J = 7.5$, 3.0 Hz, CHO), 3.83 (1H, d, $J = 14.2$ Hz, CCH$_2$H$_b$N), 3.75 (3H, s, ArOC), 3.37 (1H, dd, $J = 10.8$, 6.1 Hz, CHS), 3.21 (3H, m, CH$_2$NH + NCHf), 3.14 (1H, dd, $J = 10.3$, 4.4 Hz, NCH$_2$H$_b$), 3.03 (1H, dd, $J = 13.0$, 5.0 Hz, CH$_3$H$_b$S), 2.91 (1H, dd, $J = 13.0$, 5.0 Hz, CH$_3$H$_b$S), 2.77–2.64 (2H, m, CH$_3$H$_b$Ar + CH$_3$H$_b$Ar), 2.50 (1H, dd, $J = 10.3$, 4.4 Hz, NCH$_2$H$_b$), 2.21 (2H, t, $J = 7.3$ Hz, CH$_2$CO), 2.15–2.10 (2H, m, CH$_2$), 2.09 (3H, s, OCOCH$_3$), 1.79–1.55 (4H, m, 2 x CH$_2$), 1.45 (2H, dd, $J = 15.2$, 7.4 Hz, CH$_2$); $^{13}$C NMR $\delta$ (101 MHz, CD$_3$OD) 176.26, 172.02 (C), 166.10 (C), 159.71 (C), 144.16 (C), 131.79 (C), 130.98 (2 x CH), 125.59 (CH), 114.93 (2 x CH), 81.20 (CH), 74.63 (CH), 66.52 (CH), 63.36 (CH), 61.63 (CH), 60.12 (CH$_2$), 56.98 (CH), 55.66 (CH$_3$), 47.61 (CH$_2$), 46.92 (CH$_2$), 41.03 (CH$_2$), 37.36 (CH$_2$), 36.75 (CH$_2$), 33.57 (CH$_2$), 31.19 (CH$_2$), 29.77 (CH$_2$), 29.48 (CH$_2$), 26.77 (CH$_2$), 20.95 (CH$_3$); m/z (ESI+, MeCN) 652 ([M+Na$^+$], 25%), 630 ([M+H$^+$], 100); HRMS (ESI+, MeCN) [M+H$^+$]$^+$ found 630.3070, C$_{30}$H$_{44}$N$_7$O$_6$S requires 630.3068.
(2R,3S,4S)-1-[[1-(5S)-5-[(2-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido]ethyl)carbamoyl]-5-[(3-methyl-3H-diazirin-3-yl)propanamido]pentyl]-1H-1,2,3-triazol-4-yl]methyl)-4-hydroxy-2-[(4-methoxyphenyl)methyl]pyrrolidin-3-yl acetate 90

General procedure B was followed: to a solution of biotin diazirine azide 78 (50 mg, 0.091 mmol) in DMF (1 cm$^3$), Cu(MeCN)$_4$BF$_4$ (1.4 mg, 5 mol%) was added. The reaction was stirred for 10 min, and then TBTA (2.5 mg, 5 mol%) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (27.6 mg, 0.091 mmol) was added. The reaction was stirred overnight (18 h) at rt under an inert atmosphere. The organic layer was removed in vacuo, and the residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 90 as a colourless solid (58 mg, 75%). $R_t$ (DCM:MeOH, 4:1) = 0.6; $^1$H NMR $\delta$ (601 MHz, CD$_3$OD) 7.90 (1H, s, NCH$_3$, triazole), 7.12 (2H, d, $J$ = 8.6 Hz, 2 x ArH), 6.83 (2H, d, $J$ = 8.6 Hz, 2 x ArH), 4.64 (1H, dd, $J$ = 4.9, 2.0 Hz, CHOCOCH$_3$), 4.49 (1H, ddd, $J$ = 7.9, 5.0, 0.9 Hz, CH$_2$CH$_2$S), 4.43 (2H, t, $J$ = 6.9 Hz, CH$_2$NCH), 4.31 (1H, dd, $J$ = 7.9, 4.5 Hz, CHCHS), 4.20 (1H, dd, $J$ = 9.1, 5.3 Hz, CHNH), 4.03 (1H, d, $J$ = 14.3 Hz, CCH$_3$H$_5$N), 4.01 (1H, ddd, $J$ = 6.3, 4.4, 1.9 Hz, CHO), 3.81 (1H, d, $J$ = 14.3 Hz, CCH$_3$H$_5$N), 3.76 (3H, s, ArOCH$_3$), 3.38–3.29 (3H, m, CH$_2$NH + NCH$_3$H$_5$b), 3.38–3.29 (3H, m, CH$_2$NH + SCH$_3$), 3.13 (1H, dt, $J$ = 9.9, 5.1 Hz, NCH$_3$CH$_3$Ar), 3.02 (1H, dd, $J$ = 13.5, 5.1 Hz, CH$_3$H$_5$Ar), 2.92 (1H, dd, $J$ = 12.7, 5.0 Hz, CH$_3$H$_5$), 2.75–2.67 (2H, m, CH$_3$H$_5$S + CH$_3$H$_5$Ar), 2.47 (1H, dd, $J$ = 10.8, 4.5 Hz, NCH$_3$H$_5$), 2.26–2.10 (4H, m, 2 x CH$_2$CO), 2.09 (3H, s, COOCH$_3$), 1.99–1.90 (2H, m, CH$_2$), 1.84–1.78 (4H, m, COCHCH$_2$ + SCHCH$_2$), 1.66 (2H, t, $J$ = 7.7 Hz, CH$_2$), 1.63–1.32 (4H, m, 2 x CH$_2$), 1.01 (3H, s, CCH$_3$). $^{13}$C NMR $\delta$ (151 MHz, CD$_3$OD) 176.41 (C), 174.69 (C), 174.66 (C), 172.05
Experimental

(2R,3S,4S)-1-[[1-[(5S)-5-[(2-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido]ethyl]carbamoyl]-5-[(4-benzoylphenyl)formamido]penty]-1H-1,2,3-triazol-4-yl]methyl)-4-hydroxy-2-[(4-methoxyphenyl)methyl]pyrrolidin-3-yl acetate 91

To a solution of the biotin benzophenone azide 77 (50 mg, 0.077 mmol) in DMF (1 cm³), Cu(MeCN)₄BF₄ (1.2 mg, 5 mol%) were added. The reaction was stirred for 10 min, and then TBTA (2.0 mg, 5 mol%) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (23.4 mg, 0.077 mmol) was added. The reaction was stirred overnight (18 h) at rt under an inert atmosphere. The organic layer was removed in vacuo, and the residue was purified by column chromatography (DCM:MeOH, 9:1) to give the product 90 as a colourless solid (53 mg, 72%). Rf (DCM:MeOH, 4:1) = 0.5; ¹H NMR δ (500 MHz, CD₃OD) 8.01 (2H, d, J = 8.4 Hz, 2 x ArH), 7.90 (1H, s, NCHC, triazole), 7.84 (2H, d, J = 8.4 Hz, 2 x ArH), 7.83 – 7.77 (2H, m, 2 x ArH), 7.67 (1H, t, J = 7.5 Hz, ArH), 7.55 (2H, t, J = 7.8 Hz, 2 x ArH), 7.09
Experimental

(2H, d, J = 8.6 Hz, 2 x ArH), 6.80 (2H, d, J = 8.6 Hz, 2 x ArH), 4.65 – 4.60 (1H, m, CHOOCOCH₃), 4.50 – 4.42 (4H, m, CH₂CH₂ + CHN + CH₂NCHC), 4.26 (1H, dd, J = 7.8, 4.5 Hz, CHCHS), 4.01 – 3.94 (2H, m, CHOH + CCH₃H₂N), 3.80 – 3.73 (1H, m, CCH₂H₂N), 3.73 (3H, s, ArOCH₃), 3.52 – 3.38 (2H, m, CH₂NH), 3.36 – 3.33 (1H, m, NCH₃H₂B), 3.23 – 3.14 (2H, m, CH₂NH), 3.13 (1H, td, J = 7.2, 4.4 Hz, NCH₂CH₂Ar), 2.98 (1H, dd, J = 13.7, 4.5 Hz, CH₃H₂Ar), 2.88 (1H, dd, J = 12.7, 4.9 Hz, CH₃H₂S), 2.74 – 2.66 (1H, m, CH₃H₂Ar), 2.65 (1H, d, J = 12.7 Hz, CH₃H₂S), 2.50 – 2.41 (1H, m, NCH₃H₂B), 2.16 (2H, m, CH₂CO), 2.08 (3H, s, OCOCH₃), 2.06 – 1.81 (4H, m, 2 x CH₂), 1.63 – 1.28 (8H, m, 4 x CH₂); ¹³C NMR (126 MHz, CD₃OD) δ 197.66 (C), 176.46 (C), 174.80 (C), 172.03 (C), 169.38 (C), 166.14 (C), 159.67 (C), 141.56 (C), 138.49 (C), 138.36 (C), 134.16 (CH), 131.82 (C), 131.11 (2 x CH), 130.99 (2 x CH), 130.96 (2 x CH), 129.65 (2 x CH), 128.81 (2 x CH), 125.35 (CH), 114.93 (2 x CH), 111.39 (C), 81.25 (CH), 74.63 (CH), 66.51 (CH), 62.97 (CH), 61.73 (CH), 60.10 (CH₂), 56.83 (CH), 55.72 (CH), 55.65 (CH₂), 51.06 (CH₂), 48.33 (CH₂), 40.97 (CH₂), 40.31 (CH₂), 40.19 (CH₂), 36.80 (CH₂), 33.64 (CH₂), 32.20 (CH₂), 30.79 (CH₂), 29.43 (CH₂), 29.41 (CH₂), 26.84 (CH₂), 24.11 (CH₂), 20.96 (CH₃); m/z (ESI+, MeCN) 974 ([M+Na]+, 30%), 952 ([M+H]+, 100); HRMS (ESI+, MeCN) 974.4236 [M+Na]⁺ found, C₄₉H₆₁N₉O₃Sn requires 974.4205.

(2R,3S,4S)-1-[(1-[3-[(4-[(E)-2-[(5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido]ethyl)carbamoyl]phenyl]diazen-1-yl]phenoxy]propyl)-1H-1,2,3-triazol-4-yl]methyl]-4-hydroxy-2-[(4-methoxyphenyl)methyl]pyrrolidin-3-yl acetate 92

General procedure A was followed: to a solution of the biotin-linker-azide 53 (75 mg, 0.136 mmol) in H₂O/BuOH (1:2, 3 cm³), sodium ascorbate (6 mg, 0.03 mmol)
and copper sulphate (4 mg, 0.01 mmol) were added. The reaction was stirred for 10 min, and then TBTA (8 mg, 0.01 mmol) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (35 mg, 0.12 mmol) was added. The reaction was stirred overnight (~18 h, rt). The organic layer was removed in vacuo, and the aqueous layer was extracted with DCM (3 x 7 cm³) and EtOAc (3 x 7 cm³). The combined organic layer was dried with magnesium sulfate and concentrated in vacuo. Flash chromatography (2-10% MeOH in DCM) gave the desired product 92 as a yellow-orange solid (60 mg, 58% yield).

General procedure B was followed: to a solution of biotin-linker-azide 53 (50 mg, 0.084 mmol) in DMF (1 cm³), Cu(MeCN)₄BF₄ (1.3 mg, 5 mol%) was added. The reaction was stirred for 10 min, and then TBTA (2.2 mg, 5 mol%) was added. The mixture was stirred for a further 10 min then propargyl anisomycin 80a (27.6 mg, 0.084 mmol) was added. The reaction was stirred overnight (18 h) at rt under an inert atmosphere. The organic layer was removed in vacuo, and the residue was purified by column chromatography (2-10% MeOH in DCM) gave the desired product 92 as a yellow-orange solid (60 mg, 80% yield).

Rf (10% MeOH in DCM) = 0.19; ¹H NMR δ (500 MHz, (CD₃)₂SO) 8.55 (1H, t, J = 5.5 Hz, NH), 8.03 (2H, d, J = 8.6 Hz, 2 x ArH), 7.99 (1H, s, CH, triazole), 7.91 (2H, d, J = 9.0 Hz, 2 x ArH), 7.88 (2H, d, J = 8.6 Hz, 2 x ArH), 7.85 (1H, br s, NH), 7.80 (1H, d, J = 8.6 Hz, 2 x ArH), 7.12 (2H, d, J = 9.1 Hz, 2 x ArH), 7.07 (2H, d, J = 8.6 Hz, 2 x ArH), 6.80 (2H, d, J = 8.7 Hz, 2 x ArH), 6.28 (1H, br s, NH), 6.24 (1H, br s, NH), 4.98 (1H, d, J = 4.6 Hz, OH), 4.56 (2H, dd, J = 8.2, 4.8 Hz, CHOCOCH₃), 4.48 (2H, t, J = 7.0 Hz, CH₂NCH), 4.32 – 4.27 (1H, m, CHCH₂S), 4.24 (2H, t, J = 5.1 Hz, CH₂O), 4.13 – 4.09 (1H, m, CHCHS), 3.92 (1H, d, J = 14.2 Hz, CCH₃H₃N), 3.86 – 3.81 (1H, m, CHOH), 3.70 (3H, s, ArOCH₃), 3.66 (1H, d, J = 14.2 Hz, CCH₃H₃N), 3.36 (2H, br q, J = 6.1 Hz, CH₂NH), 3.26 (2H, br q, J = 6.1 Hz, CH₂NH), 3.10 – 3.05 (1H, m, CHS), 3.05 – 3.01 (1H, m, NCCH₃H₃), 2.88 (1H, dd, J = 12.7, 4.9 Hz, CH₃H₃S), 2.80 (2H, m, CH₃H₃Ar, CHCH₃Ar), 2.58 – 2.54 (2H, m, CH₃H₃S, CH₃H₃Ar), 2.09 (2H, t, J = 7.3 Hz, CH₂CO), 2.06 – 2.04 (1H, m, NCCH₃H₃), 2.02 (3H, s, OCOCCH₃), 1.67 – 1.59 (2H, m, CH₂), 1.57 – 1.30 (6H, m, 3 x CH₂); ¹³C NMR δ (126 MHz, DMSO) 172.32 (C), 169.48 (C), 165.56 (C), 162.50 (C), 161.35 (C), 157.39 (C), 153.36 (C), 146.27
(C), 143.27 (C), 135.94 (C), 130.82 (C), 129.60 (2 x CH), 128.24 (2 x CH), 124.63 (2 x CH), 123.47 (CH), 121.77 (2 x CH), 115.07 (2 x CH), 113.51 (2 x CH), 79.43 (CH), 72.68 (CH), 65.05 (CH₂), 64.54 (CH), 60.90 (CH), 59.12 (CH), 55.87 (CH₂), 55.15 (CH), 48.41 (CH₃), 47.20 (CH₂), 46.21 (CH₂), 39.60 (CH₂), 38.12 (CH₂), 35.13 (CH₂), 32.33 (CH₂), 29.23 (CH₂), 27.96 (CH₂), 27.88 (CH₂), 25.04 (CH₂), 20.54 (CH₃); m/z (ESI, +) 1815 ([2M+Na]⁺, 9%), 919 ([M+Na]⁺, 35), 897 ([M+H]⁺, 100); HRMS (ESI, +) found [M+H]⁺ 897.4087, C₄₅H₅₇N₁₀O₈S₁ requires 897.4076.
5.4 Experimental for Chapter 3

2-(4-bromophenyl) ethyl iodide 110

To a stirred solution of 2-(4-bromophenyl) ethyl alcohol 109 (2.50 g, 12.4 mmol), and triethylamine (2.26 cm³, 16.2 mmol) in DCM (25 cm³) at 0 °C, methanesulfonyl chloride (1.15 cm³, 14.8 mmol) was added. The suspension was stirred at rt for 2 h. The reaction mixture was washed with brine (2 x 25 cm³), dried with magnesium sulfate and concentrated in vacuo to yield the intermediate mesylate 109a an orange/red oil (3.21 g); \(^{1}H\) NMR \(\delta\) (400 MHz, CDCl\(_3\)) 7.45 (2H, d, \(J = 8.4 \text{ Hz, ArH}\)), 7.12 (2H, d, \(J = 8.4, \text{ ArH}\)), 4.39 (2H, t, \(J = 6.8, \text{ CH}_2\)), 3.01 (2H, t, \(J = 6.8, \text{ CH}_2\)), 2.89 (3H, s, SO\(_2\text{CH}_3\)).

Sodium iodide (1.86 g, 12.4 mmol) was added to the oil 109a in 2-butanone (40 cm³), and the reaction mixture was heated under reflux (82 °C) for 4.5 h. The reaction mixture was diluted with EtOAc (50 cm³), and washed with H\(_2\)O (2 x 25 cm³), 10% aqueous sodium thiosulfate (2 x 25 cm³), and brine (2 x 25 cm³). The organic layer was dried with magnesium sulfate and concentrated in vacuo. Flash chromatography (10% EtOAc in hexane) gave the desired product 110 (3.02 g, 78% yield) as a colourless oil; \(R_f\) (10% EtOAc in hexane) = 0.75; \(^{1}H\) NMR \(\delta\) (400 MHz, CDCl\(_3\)) 7.44 (2H, d, \(J = 6.7 \text{ Hz, ArH}\)), 7.07 (2H, d, \(J = 6.7 \text{ Hz, ArH}\)), 3.32 (2H, t, \(J = 6.7 \text{ Hz, CH}_2\)), 3.13 (2H, t, \(J = 7.4 \text{ Hz, CH}_2\)); \(^{13}C\) NMR \(\delta\) (101 MHz, CDCl\(_3\)) 139.56 (C), 131.88 (2 x CH), 130.21 (2 x CH), 120.90 (C), 39.64 (CH\(_2\)), 5.14 (CH\(_2\)).

All spectroscopic data in good agreement with the literature.\(^{190}\)
Diethyl 2-acetamido-2-[2-(4-bromophenyl)ethyl] maleonate 111

A solution of diethyl acetamidomaleonate (2.87 g, 13.2 mmol) in DMF (5.5 cm³) was added dropwise to a suspension of sodium hydride (60% dispersion in mineral oil, 572 mg, 14.3 mmol) in DMF (11 cm³) at 0 °C. After stirring for 45 min, 2-(4-bromophenyl)ethyl iodide 110 (3.02 g, 9.73 mmol) was added dropwise to the mixture at 0 °C. The reaction mixture was stirred at rt (1 h) and 60°C (2 h), poured into ice water (50 cm³), and extracted with EtOAc (2 x 25 cm³). The organic layer was dried with magnesium sulfate and concentrated in vacuo. Flash chromatography (4:1 hexane:EtOAc) gave the desired product 111 (3.8 g, 98%); Rf (4:1 Hexane:EtOAc) = 0.08; ¹H NMR δ (400 MHz, CDCl₃) 7.38 (2H, d, J = 8.4 Hz, ArH), 7.02 (2H, d, J = 8.4 Hz, ArH), 6.75 (1H, s, NH), 4.30–4.12 (4H, m, 2 x CH₃CH₂O), 2.66 (2H, dd, J = 9.7, 6.6 Hz, ArCH₂CH₂), 2.44 (2H, dd, J = 9.7, 6.6 Hz, ArCH₂CH₂), 1.99 (3H, s, OAc), 1.24 (6H, t, J = 7.1 Hz, 2 x CH₃CH₂O); ¹³C NMR δ (101 MHz, CDCl₃) 169.25 (C), 168.09 (C), 139.70 (C), 131.56 (2 x CH), 130.35 (2 x CH), 120.01 (C), 110.12 (C), 66.43 (C), 62.80 (2 x CH₂), 33.38 (CH₂), 29.75 (CH₂), 23.14 (CH₃), 14.13 (2 x CH₃).

All spectroscopic data in good agreement with the literature.

2-acetamido-2-[2-(4-bromophenyl)ethyl]propane-1, 3-diol diacetate 112

To a stirred solution of diethyl 2-acetamido-2-[2-(4-bromophenyl)ethyl] maleonate 111 (3.7995 g, 9.493 mmol) and K₂HPO₄ (2.5 g) in EtOH (45 cm³) cooled to 0 °C, a solution of NaBH₄ (2.4 M in EtOH, 1.21 cm³, 29 mmol) was added dropwise. The mixture was warmed to rt and stirred for 12 h. The reaction was quenched with Na₂SO₃ (100 cm³; sat aq), and the reaction mixture was filtered through Celite. The mixture was concentrated in vacuo to give the desired intermediate diol 111b (1.3 g, 51%); ¹H
Experimental

NMR (400 MHz, CDCl\textsubscript{3}) \(\delta\) 7.39 (1H, d, \(J = 8.4\) Hz, Ar\(H\)), 7.07 (1H, d, \(J = 8.4\) Hz, Ar\(H\)), 3.83 (2H, d, \(J = 11.5\) Hz, CH\textsubscript{2}OH), 3.62 (2H, d, \(J = 11.5\), CH\textsubscript{2}OH), 2.62–2.56 (2H, m, CH\textsubscript{2}CH\textsubscript{2}Ar), 2.00 (2H, s, NH\textsubscript{2}), 1.26 (2H, m, CH\textsubscript{2}CH\textsubscript{2}Ar).

To a stirred solution of 2-amino-2-[2-(4-bromophenyl)ethyl]propane-1,3-diol \textbf{111b} (1.33 g, 4.85 mmol) in THF (11 cm\textsuperscript{3}), acetic anhydride (1.51 cm\textsuperscript{3}, 16.0 mmol), triethylamine (2.23 cm\textsuperscript{3}, 16.0 mmol) and DMAP (20 mg, 1.6 mmol) were added. After 15 mins, the reaction was quenched with water (30 cm\textsuperscript{3}) and extracted with EtOAc (2 x 30 cm\textsuperscript{3}). The organic layer was washed with brine, dried with magnesium sulfate and concentrated in vacuo. Flash chromatography (EtOAc) gave the desired triacetate \textbf{112} (1.3 g, quant.); \(R_t\) (EtOAc) = 0.15; \(\textsuperscript{1}H\) NMR \(\delta\) (400 MHz, CDCl\textsubscript{3}) 7.29 (2H, d, \(J = 7.2\) Hz, Ar\(H\)), 7.18 (2H, d, \(J = 7.2\) Hz, Ar\(H\)), 6.17 (1H, s, NHAc), 4.37 (4H, q, \(J = 11.2\) Hz, 2 x CH\textsubscript{2}OAc), 2.69–2.56 (2H, m, ArCH\textsubscript{2}CH\textsubscript{2}H), 2.29–2.14 (2H, m, ArCH\textsubscript{2}CH\textsubscript{2}H), 2.08 (6H, s, 2 x OAc), 1.98 (3H, s, NH\textsubscript{Ac}); \(\textsuperscript{13}C\) NMR \(\delta\) (101 MHz, CDCl\textsubscript{3}) 171.04 (2 x C), 170.97 (C), 141.51(C), 128.58(2 x CH), 126.26 (2 x CH), 64.83 (2 x CH\textsubscript{2}), 58.37 (C), 33.87 (CH\textsubscript{2}), 29.83 (CH\textsubscript{2}), 24.26 (CH\textsubscript{3}), 21.06 (2 x CH\textsubscript{3}).

All spectroscopic data in good agreement with the literature.\textsuperscript{190}

\textbf{2-(4-Iodophenyl)ethan-1-ol 114}

To a stirred solution of 2-(4-aminophenyl)ethan-1-ol \textbf{113} (2.00 g, 14.6 mmol) in hot water (10 cm\textsuperscript{3}) was added H\textsubscript{2}SO\textsubscript{4} (1.35 cm\textsuperscript{3}; 4.67 M), the reaction was then cooled to 0\textdegreeC in an ice bath. A second addition H\textsubscript{2}SO\textsubscript{4} (6.35 cm\textsuperscript{3}; 3.83 M) was added dropwise slowly to maintain the temperature at 0-5 °C, after which sodium nitrite (1.30 g, 15.3 mmol) in H\textsubscript{2}O (5 cm\textsuperscript{3}) was added dropwise and the reaction was stirred for 30 min. Potassium iodide (8.5 g, 51.2 mmol) in H\textsubscript{2}O (10 cm\textsuperscript{3}) was added dropwise and the reaction was stirred for 2 h and warmed to rt. The reaction was heated to 60 °C for 30 min, cooled to rt and then extracted with EtOAc (3 x 200 cm\textsuperscript{3}). The combined organic layer was washed with sodium thiosulfate (2 x 100 cm\textsuperscript{3}; sat aq), brine (100 cm\textsuperscript{3}), dried
Experimental

(MgSO₄) and concentrated in vacuo. The oil was purified by column chromatography (EtOAc:hexane, 3:1) to give the desired compound 114 as a colourless oil (3.19 g, 88%). **Rf** (EtOAc:hexane, 3:1) = 0.17; **IR** (neat, cm⁻¹) 3325 (OH); **¹H NMR** δ (500 MHz, CDCl₃) 7.63 (2H, d, J = 8.3 Hz, ArH), 6.99 (2H, d, J = 8.3 Hz, ArH), 3.85 (2H, t, J = 6.5 Hz, ArCH₂C₂H₂OH), 2.81 (2H, t, J = 6.5 Hz, ArCH₂CH₂OH); **¹³C NMR** δ (126 MHz, CDCl₃) 138.39 (C), 137.77 (2 x CH), 131.25 (2 x CH), 91.82 (C), 63.53 (CH₂), 38.80 (CH₂); m/z (EI +, MeCN) 247 ([M]+, 14), 77 (100); **HRMS** (EI+, MeCN) [M]+ found 247.9703, C₈H₉O⁻I₁ requires 247.96927.

**1-Iodo-4-(2-iodoethyl)benzene 115**

![1-Iodo-4-(2-iodoethyl)benzene 115](image)

To 2-(4-iodophenyl)ethan-1-ol 114 (3.00 g, 12.1 mmol) in DCM (40 cm³) at 0 °C was added PPh₃ (6.34 g, 24.1 mmol), imidazole (1.73 g, 25.4 mmol), iodine (6.14 g, 24.1 mmol), and the reaction was stirred for 2 h at rt under an inert atmosphere. The reaction mixture was quenched with Na₂SO₃ (100 cm³; sat aq), and extracted with DCM (3 x 100 cm³). The combined organic layer was washed with brine (100 cm³), dried (MgSO₄) and concentrated in vacuo. The residue was dissolved in hexane (150 cm³) and cooled in an ice bath and the precipitate formed was filtered through a celite/silica plug. The filtrate was concentrated in vacuo to give the product 115 as a light yellow oil (3.85 g, 89%). **Rf** (EtOAc:hexane, 9:1) = 0.68; **IR** (neat, cm⁻¹) 1483 (C=C); **¹H NMR** δ (500 MHz, CDCl₃) 7.64 (2H, d, J = 8.3 Hz, ArH), 6.95 (2H, d, J = 8.3 Hz, ArH), 3.32 (2H, t, J = 7.6 Hz, ArCH₂CH₂I), 3.12 (2H, t, J = 7.6 Hz, ArCH₂CH₂I); **¹³C NMR** δ (126 MHz, CDCl₃) 140.23 (C), 137.78 (2 x CH), 130.51 (2 x CH), 92.32 (C), 39.75 (CH₂), 4.99 (CH₂); m/z (EI+, MeCN) 357 ([M]+, 24%), 230 ([M-I]+, 100); **HRMS** (EI+, MeCN) [M]+ found 357.87090, C₈H₈I⁻I₂ requires 357.87101.
Experimental

Diethyl 2-acetamido-2-[2-(4-iodophenyl)ethyl]propanedioate 116

To a stirred suspension of NaH (80.5 mg, 3.35 mmol) in DMF (15 cm$^3$) at 0 °C was added diethyl acetamidomaleonate (874 mg, 4.02 mmol) in DMF (5 cm$^3$) dropwise. The reaction mixture was stirred for 1 h at rt under N$_2$, after which 1-iodo-4-(2-iodoethyl)benzene 115 (1.20 g, 3.35 mmol) in DMF (15 cm$^3$) was added dropwise. The reaction mixture was stirred for 3 h at rt and 2 h at 50 °C, after which the solution was poured into iced water (250 cm$^3$), and extracted with EtOAc (3 x 100 cm$^3$). The combined organic layer was washed with brine (5 x 100 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo. The solid was purified by column chromatography (EtOAc:hexane, 1:1) to give the desired compound 116 as a colourless solid (1.23 g, 82%). $\text{Rf}$ (EtOAc:hexane, 1:1) = 0.54; mp pending, IR (neat, cm$^{-1}$) 3360 (NH), 1739 (CO), 1680 (CO); $^1\text{H NMR}$ $\delta$ (500 MHz, CDCl$_3$) 7.58 (2H, d, $J = 8.3$ Hz, ArH), 6.90 (2H, d, $J = 8.3$ Hz, ArH), 6.74 (1H, s, NHCOCH$_3$), 4.27 – 4.15 (4H, m, 2 x CH$_3$CH$_2$O), 2.69 – 2.62 (2H, m, ArCH$_2$CH$_2$C), 2.45 – 2.39 (2H, m, ArCH$_2$CH$_2$C), 1.99 (3H, s, NHCOCH$_3$), 1.25 (6H, t, $J = 7.1$ Hz, 2 x CH$_3$CH$_2$O); $^{13}\text{C NMR}$ $\delta$ (126 MHz, CDCl$_3$) 169.24 (C), 168.09 (2 x C), 140.41 (C), 137.56 (2 x CH), 130.70 (2 x CH), 91.26 (C), 66.45 (C), 62.80 (2 x CH$_2$), 33.35 (CH$_2$), 29.86 (CH$_2$), 23.14 (CH$_3$), 14.13 (2 x CH$_3$); m/z (ESI+, MeCN) 486 ([M+K]$^+$, 10%), 470 ([M+Na]$^+$, 100), 448 ([M+H]$^+$, 50); HRMS (ESI+, MeCN) [M+H]$^+$ found 448.0615, C$_{17}$H$_{23}$INO$_5$ requires 448.0615.

N-{1,3-Dihydroxy-2-[2-(4-iodophenyl)ethyl]propan-2-yl}acetamide

To diester 115 (633 mg, 1.42 mmol) in ethanol (40 cm$^3$, anhydrous) was added K$_2$HPO$_4$ (1.95 g, 11.2 mmol) and then NaBH$_4$ (273 mg, 7.22 mmol). The reaction solution was stirred overnight (18 h) at rt under N$_2$. The solution was poured slowly
into a solution of EtOAc (100 cm$^3$) and NH$_4$Cl (100 cm$^3$; sat aq). The organic layer was separated, the aqueous layer was extracted with EtOAc (3 x 100 cm$^3$), and the combined organic layer was washed with brine (20 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo. The solid was purified by column chromatography (DCM:MeOH, 19:1) to give the desired compound as a colourless solid (0.514 g, quant.). \( R_f \) (DCM:MeOH, 19:1) = 0.12; \( \text{IR} \) (neat, cm$^{-1}$) 3292 (OH, NH), 1645 (CO);

$^1$H NMR \( \delta \) (500 MHz, CD$_3$OD) 7.58 (2H, d, \( J = 8.3 \) Hz, ArH), 7.01 (2H, d, \( J = 8.3 \) Hz, ArH), 3.76 (2H, d, \( J = 11.2 \) Hz, 2 x CH$_2$H$_2$OH), 3.67 (2H, d, \( J = 11.2 \) Hz, 2 x CH$_2$H$_2$OH), 2.59 – 2.52 (2H, m, ArCH$_2$), 2.01 – 1.94 (2H, m, ArCH$_2$CH$_2$C), 1.97 (3H, s, NHCOCH$_3$); $^{13}$C NMR \( \delta \) (126 MHz, CD$_3$OD) 173.99 (C), 143.73 (C), 138.51 (2 x CH), 131.71 (2 x CH), 91.22 (C), 63.89 (2 x CH$_2$), 62.44 (C), 33.93 (CH$_2$), 30.07 (CH$_2$), 23.40 (CH$_3$); \( m/z \) (ESI+, MeCN) 386 ([M+Na]$^+$, 50%), 364 ([M+H]$^+$, 100); HRMS (ESI+, MeCN) [M+Na]$^+$ found 386.0227, C$_{13}$H$_{18}$INO$_3$Na requires 386.0224.

### 2-[(Acetyloxy)methyl]-2-acetamido-4-(4-iodophenyl)butyl acetate 116

To a stirred solution of previously-made diol (178 mg, 0.489 mmol) in THF (10 cm$^3$) was added acetic anhydride (277 µL, 2.94 mmol), triethylamine (409 µL, 2.94 mmol), and DMAP (59.7 mg, 0.489 mmol) and the reaction mixture was stirred overnight (18 h) at rt under N$_2$. The reaction was quenched with H$_2$O (20 cm$^3$) and NaHCO$_3$ (20 cm$^3$; sat aq), and extracted with EtOAc (3 x 40 cm$^3$). The combined organic layer was washed with brine (20 cm$^3$), dried (MgSO$_4$) and concentrated in vacuo. The residue was purified by column chromatography (EtOAc:hexane, 3:1) to give the desired product 116 as a colourless solid (0.153 g, 70%). \( R_f \) (EtOAc:hexane, 3:1) = 0.37; \( \text{IR} \) (neat, cm$^{-1}$) 3306 (NH), 1741 (CO), 1660 (CO); $^1$H NMR \( \delta \) (500 MHz, CDCl$_3$) 7.59 (2H, d, \( J = 8.3 \) Hz, ArH), 6.94 (2H, d, \( J = 8.3 \) Hz, ArH), 5.66 (1H, s, NHAc), 4.32 (4H, s, CH$_2$OAc), 2.58 – 2.52 (2H, m, ArCH$_2$CH$_2$C), 2.21 – 2.15 (2H, m, ArCH$_2$CH$_2$C), 2.09 (6H, s, 2 x COOCH$_3$), 1.97 (3H, s, NHCOCH$_3$); $^{13}$C NMR \( \delta \) (126 MHz, CDCl$_3$) 170.96 (2 x C), 170.22 (C), 141.16 (C), 137.70 (2 x CH), 130.64 (2 x CH), 91.29 (C), 30.07 (CH$_2$), 23.40 (CH$_3$); \( m/z \) (ESI+, MeCN) 410 ([M+Na]$^+$, 42%); HRMS (ESI+, MeCN) [M+Na]$^+$ found 410.0192, C$_{17}$H$_{20}$INO$_3$Na requires 410.0190.
Experimental

64.79 (2 x CH₂), 58.38 (C), 33.72 (CH₂), 29.47 (CH₂), 24.34 (CH₃), 20.98 (2 x CH₃);

m/z (ESI+, MeCN) 486 ([M+K]⁺, 20%), 470 ([M+Na]⁺, 100), 448 ([M+H]⁺, 40);

HRMS (ESI+, MeCN) [M+H]⁺ found 448.0618, C₁₇H₂₂INO₅ requires 448.0615.

6-Heptyn-1-ol 118

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\begin{array}{c}
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\text{CH}_2
\end{array} \\
\text{OH}
\end{array}
\]

To a stirred solution of LiAlH₄ (1.76 cm³, 4.22 mmol, 2.4 M in THF) in THF (10 cm³) at 0 °C, 6-heptynoic acid 117 (250 µL, 1.98 mmol) was added dropwise. The reaction mixture was allowed to warm to rt and stirred overnight (~16 h). The reaction was cooled to 0 °C and sodium hydroxide (2.5 cm³; 2 M) was added. Water (25 cm³) was added to the reaction mixture which was extracted with EtOAc (3 x 25 cm³). The combined organic layer was dried using magnesium sulfate and reduced in vacuo. Flash chromatography (10-20% EtOAc in hexane) gave the desired product 118 as a colourless oil (187 mg, 85% yield); Rₚ (10% EtOAc in hexane) = 0.5; \(^1\)H NMR δ (400 MHz, CDCl₃) 3.66 (2H, t, J = 6.4 Hz, CH₂OH), 2.21 (2H, td, J = 6.8, 2.6 Hz, CH₂CCH), 1.94 (1H, t, J = 2.6 Hz, CHCCH₂), 1.71–1.32 (6H, m, 3 x CH₂); \(^1\)C NMR δ (101 MHz, CDCl₃) 84.56 (C), 68.46 (CH), 62.95 (CH₂), 32.36 (CH₂), 28.36 (CH₂), 25.03 (CH₂), 18.51 (CH₂).

\(^1\)H spectroscopic data in good agreement with the literature.²²¹

2-[(Acetyloxy)methyl]-2-acetamido-4-[4-(7-hydroxyhept-1-yn-1-yl)phenyl]butyl acetate 119

\[
\begin{array}{c}
\text{AcO}
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\]

Aryl iodide 116 (490 mg, 1.11 mmol) was dissolved in THF (20 cm³) and triethylamine (10 cm³, 71 mmol). 5 Å molecular sieves (0.5 g) were added and the solution was degassed. To this was added PdCl₂(PPh₃)₂ (39.2 mg, 5 mol%), CuI (0.0213 g, 10
mol%) and was stirred for 10 min. Heptyn-1-ol 118 (0.304 g, 1.34 mmol) in THF (5 cm³) was added dropwise, and the reaction was stirred at 45 °C, in the dark, overnight (18 h) under N₂. The reaction mixture was quenched with NH₄Cl (100 cm³; sat aq), and extracted with DCM (3 x 100 cm³). The combined organic layer was washed with brine (100 cm³), dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 19:1) to give the product 119 as a colourless solid (0.412 g, 85%). Rᵣ (DCM:MeOH, 19:1) = 0.15; IR (neat, cm⁻¹) 3300 (NH, OH), 1743 (CO), 1662 (CO); ¹H NMR δ (500 MHz, CDCl₃) 7.30 (2H, d, J = 8.2 Hz, 2 x ArH), 7.09 (2H, d, J = 8.2 Hz, 2 x ArH), 5.66 (1H, s, NHCOCH₃), 4.33 (4H, s, 2 x CH₂OCOCH₃), 3.66 (2H, dd, J = 11.8, 6.4 Hz, CH₂OH), 2.62 – 2.55 (2H, m, CH₂Ar), 2.41 (2H, t, J = 7.0 Hz, CCH₂), 2.22 – 2.15 (2H, m, CH₂CH₂Ar), 2.09 (6H, s, 2 x OCOC₂H₃), 1.96 (3H, s, NHCOCH₃), 1.67 – 1.59 (4H, m, 2 x CH₂), 1.57 – 1.50 (2H, m, CH₂); ¹³C NMR δ (126 MHz, CDCl₃) 170.97 (2 x C), 170.21 (C), 140.99 (C), 131.83 (2 x CH), 128.41 (2 x CH), 121.92 (C), 89.81 (C), 80.76 (C), 64.79 (2 x CH₂), 63.04 (CH₂), 58.40 (C), 33.64 (CH₂), 32.45 (CH₂), 29.75 (CH₂), 28.68 (CH₂), 25.22 (CH₂), 24.33 (CH₃), 20.98 (2 x CH₃), 19.54 (CH₂); m/z (ESI+, MeCN) 470 ([M+K]⁺, 10), 454 ([M+Na]⁺, 100), 432 ([M+H]⁺, 50); HRMS (ESI+, MeCN) [M+H]⁺ found 432.2384, C₂₄H₃₄NO₆ requires 432.2381.

2-[(Acetyloxy)methyl]-2-acetamido-4-[4-(7-hydroxyheptyl)phenyl]butyl acetate 120

Alkynyl alcohol 119 (359 mg, 0.831 mmol) and 10 % Pd/C (80.0 mg, 0.752 mmol) in DCM (50 cm³) was stirred overnight (18 h) at rt under an atmosphere of H₂. The reaction mixture was filtered through a pad of celite and the celite was washed with DCM (3 x 100 cm³). The solution was concentrated in vacuo to give the desired product 120 as a colourless solid (0.343 g, 95%). Rᵣ (DCM:MeOH, 19:1) = 0.17; IR (neat, cm⁻¹) 3302 (NH, OH), 1743 (CO), 1660 (CO); ¹H NMR δ (500 MHz, CDCl₃) 7.09 (4H, m, 4 x ArH), 5.63 (1H, s, NHCOCH₃), 4.35 (4H, s, 2 x CH₂OCOCH₃), 3.63

Experimental

(2H, t, \( J = 5.2 \) Hz, \( \text{CH}_2\text{OH} \)), 2.62 – 2.52 (4H, m, 2 x \( \text{CH}_2\text{Ar} \)), 2.24 – 2.15 (2H, m, \( \text{CH}_2\text{C} \)), 2.09 (6H, s, 2 x \( \text{OCOCH}_3 \)), 1.95 (3H, s, \( \text{NHOCH}_3 \)), 1.65 – 1.54 (4H, m, 2 x \( \text{CH}_2 \)), 1.36 – 1.32 (6H, m, 3 x \( \text{CH}_2 \)) \( \text{C}^{13} \) NMR \( \delta \) (126 MHz, CDCl\(_3\)) 170.98 (2 x C), 170.15 (C), 140.79 (C), 138.60 (C), 128.70 (2 x CH), 128.37 (2 x CH), 64.83 (2 x \( \text{CH}_2 \)), 63.20 (CH\(_2\)), 58.43 (C), 35.63 (CH\(_2\)), 33.89 (CH\(_2\)), 32.92 (CH\(_2\)), 31.59 (CH\(_2\)), 29.41 (2 x CH\(_2\)), 29.37 (CH\(_2\)), 25.81 (CH\(_3\)), 24.32 (CH\(_3\)), 20.99 (2 x CH\(_3\)); \text{m/z} \text{ (ESI+}, \text{MeCN)} 458 ([M+Na]+, 32%), 436 ([M+H]+, 54), 376 (100); \text{HRMS (ESI+}, \text{MeCN)} [M+H]+ found 436.2694, \( \text{C}_{24}\text{H}_{38}\text{NO}_6 \) requires 436.2700.

\text{N-(1,3-dihydroxy-2-[2-[4-(oct-7-yn-1-yl)phenyl]ethyl]propan-2-yl)acetamide 123}

Alkyl alcohol 120 (50.0 mg, 0.115 mmol) was dissolved in DCM:DMSO (2:1, 10 cm\(^3\)), triethylamine (0.0539 cm\(^3\), 0.574 mmol) and SO\(_3\)-py (0.0731 g, 0.459 mmol) were added to the reaction mixture and stirred for 1 h at rt. The reaction was quenched with H\(_2\)O (10 cm\(^3\)) and extracted with EtOAc (4 x 20 cm\(^3\)). The combined organic layer was dried (MgSO\(_4\)), concentrated \text{in vacuo} to give the aldehyde 121 as a colourless oil (50 mg, quant.) which was used immediately in the next step. The aldehyde oil was dissolved in MeOH (10 cm\(^3\), anhydrous), potassium carbonate (0.128 g, 0.923 mmol) and Ohira-Bestmann reagent (88.6 mg, 0.461 mmol) was added with stirring under N\(_2\). The reaction was left to stir overnight (18 h). The reaction mixture was quenched with NaHCO\(_3\) (20 cm\(^3\), sat aq), extracted with EtOAc (3 x 25 cm\(^3\)). The combined organic layer was washed with brine (50 cm\(^3\), sat aq), dried (Na\(_2\)SO\(_4\)) and concentrated \text{in vacuo}. The residue was used as the crude in the next step – due to mixture of protected and unprotected hydroxyl groups.
2-Amino-2-{2-[4-(oct-7-yn-1-yl)phenyl]ethyl}propane-1,3-diol 100

Crude alkyne mixture 123/124 (74.7 mg, 0.216 mmol) and LiOH•H2O (71.7 mg, 1.71 mmol) were dissolved in MeOH:THF:H2O (2:1:2, 10 cm3) and stirred for 5 h at rt under N2. The reaction mixture was diluted with EtOAc (20 cm3), washed with EtOAc (20 cm3), dried (MgSO4) and concentrated in vacuo. The residue was purified by column chromatography (DCM:MeOH, 19:1) to give the product 100 as a colourless solid (12.1 mg, 97%); Rf (DCM:MeOH:NH3, 19:1:1) = 0.2; 1H NMR δ (601 MHz, CDCl3) 7.13 – 7.05 (4H, m, 4 x ArH), 3.57 (4H, dd, J = 55.8, 10.7 Hz, 2 x CH2OH), 2.64 – 2.59 (2H, m, CH2Ar), 2.59 – 2.54 (2H, m, CH2Ar), 2.17 (2H, td, J = 7.1, 2.6 Hz, CHJCCCH), 1.93 (1H, t, J = 2.7 Hz, CCH), 1.75 – 1.69 (2H, m, CH2), 1.60 (1H, p, J = 7.7 Hz, CH2), 1.52 (1H, p, J = 7.1 Hz, CH2), 1.47 – 1.38 (2H, m, CH2), 1.38 – 1.30 (2H, m, CH2); 13C NMR δ (151 MHz, CDCl3) 140.57 (C), 139.20 (C), 128.68 (2 x ArCH), 128.26 (2 x ArCH), 84.86 (C), 68.26 (CH), 67.67 (2 x CH2), 56.15 (C), 37.47 (CH2), 35.57 (CH2), 31.49 (CH2), 29.23 (CH2), 28.89 (CH2), 28.72 (CH2), 28.54 (CH2), 18.52 (CH2); m/z (ESI+, MeCN) 304 ([M+H]+, 100%); HRMS (ESI+, MeCN) [M+H]+ found 304.2286, C19H30NO2 requires 304.2271.

Oct-7-yn-1-ol 129

Ethylene diamine (9 cm3) was cooled to 0 °C and charged with NaH in one portion (0.8 g, 20 mmol, 60% dispersion in mineral oil). The mixture was stirred at 0 °C for 5 min, then 1 hr at room temperature, then 65 °C for 1 hr. The mixture was then cooled to 45 °C before addition of 3-octyn-1-ol (630 mg, 5 mmol) dropwise over 2 minutes. After warming back to 65 °C the mixture was stirred at that temperature for 1 hour then cooled to 0 °C and water (8 cm3) was slowly added followed by slow addition of
HCl (10 cm$^3$; 1 M). A further portion of HCl (10 cm$^3$; 1 M) was then added and organics were extracted with Et$_2$O (3 x 25 cm$^3$) and washed with HCl (10 cm$^3$; 1 M), brine (10 cm$^3$), dried (MgSO$_4$), filtered through a silica plug topped with celite using Et$_2$O (2 x 20 cm$^3$) to elute and concentrated in vacuo to provide 7-octyn-1-ol as a yellow oil (630 mg, quant.) of a sufficient purity to advance without further purification. 

**Experimental**

**1H NMR** $\delta$ (601 MHz, CDCl$_3$) 3.64 (2H, t, $J = 6.6$ Hz, CH$_2$OH), 2.19 (2H, td, $J = 7.1, 2.6$ Hz, CH$_2$CCH), 1.94 (1H, t, $J = 2.6$ Hz, CHCCH$_2$), 1.61 – 1.50 (4H, m, 2 x CH$_2$), 1.47 – 1.36 (4H, m, 2 x CH$_2$);

**13C NMR** $\delta$ (151 MHz, CDCl$_3$) 84.72 (C), 68.33 (CH), 65.99 (CH$_2$), 63.05 (CH$_2$), 32.75 (CH$_2$), 28.53 (CH$_2$), 28.52 (CH$_2$), 25.38 (CH$_2$), 18.47 (CH$_2$); m/z (EI) 126 ([M], 100%).

All spectroscopic data in good agreement with the literature.$^{222}$

**8-Iodoocct-1-yn-1-yl)trimethylsilane 130**

$n$-BuLi (4.39 cm$^3$, 10.9 mmol, 2.5 M in hexane) was added at -78 °C under nitrogen to a mixture of octyn-1-ol 129 (630 mg, 4.99 mmol) in dry THF (5 cm$^3$), followed by DMAP (152 mg, 1.24 mmol). After the mixture was stirred for 1 h, TMSCl (2.22 cm$^3$, 10.9 mmol) was added, and was gradually warmed to rt and stirred for 2 h. Then, HCl (5 cm$^3$; 1 M) was added and the reaction mixture was stirred vigorously for 30 min. The reaction was diluted with EtOAc (20 cm$^3$), and the organic layer was separated. The aqueous layer was extracted with EtOAc (3 x 20 cm$^3$), and the combined organic extract was washed with NaHCO$_3$ (50 cm$^3$; sat aq) and brine (50 cm$^3$). The reaction was dried (MgSO$_4$) and concentrated in vacuo to give the residue, which was purified by column chromatography (hexane:EtOAc, 4:1) to give the desired intermediate TMS-protected alkynol as a colourless oil (790 mg, 80%). 

**Rf** (hexane:EtOAc, 4:1) = 0.24; **1H NMR** $\delta$ (500 MHz, CDCl$_3$) 3.64 (2H, t, $J = 6.6$ Hz, CH$_2$OH), 2.22 (2H, t, $J = 7.1$ Hz, CCH$_2$), 1.63 – 1.49 (4H, m, 2 x CH$_2$), 1.46 – 1.33 (4H, m, 2 x CH$_2$), 0.14
Experimental

(9H, s, 3 x CH₃); ¹³C NMR δ (126 MHz, CDCl₃) 107.66 (C), 84.58 (C), 63.08 (CH₂), 32.77 (CH₂), 28.67 (2 x CH₂), 25.35 (CH₂), 19.92 (CH₂), 0.32 (3 x CH₃).

To the TMS-alkynol (486 mg, 2.45 mmol) in dry THF (20 cm³) at 0 °C was added triphenylphosphine (0.964 g, 3.67 mmol) followed by imidazole (250 mg, 3.67 mmol). Then, iodine (932 mg, 3.67 mmol) was added in one portion to the reaction mixture, and stirred for 3 hr. The reaction mixture was diluted with DCM (30 cm³), and the reaction was washed with Na₂S₂O₃ (50 cm³, 10% aq), followed with brine (50 cm³), dried (MgSO₄) and concentrated in vacuo. The resulting oil was triturated in pentane (100 cm³) and filtered through a celite pad to give the product as a colourless oil (725 mg, 96%). Rf (hexane:EtOAc, 9:1) = 0.6; ¹H NMR δ (500 MHz, CDCl₃) 3.19 (2H, t, J = 7.0 Hz, CH₂I), 2.22 (2H, t, J = 7.1 Hz, CCH₂), 1.88 – 1.79 (2H, m, CH₂), 1.56 – 1.49 (2H, m, CH₂), 1.46 – 1.36 (4H, m, 2 x CH₂), 0.15 (9H, s, 3 x CH₃); ¹³C NMR δ (126 MHz, CDCl₃) 107.46 (C), 84.72 (C), 33.49 (CH₂), 30.10 (CH₂), 27.77 (CH₂), 19.91 (CH₂), 7.10 (CH₂), 0.33 (3 x CH₃).

All spectroscopic data in good agreement with the literature.

2-[(acetyloxy)methyl]-2-acetamido-4-{4-[8-(trimethylsilyl)oct-7-yn-1-yl][phenyl]butyl acetate 131

To a suspension of zinc powder (243 mg, 3.72 mmol) in dimethylacetamide (1 cm³) was added catalytic iodine (47.2 mg, 0.186 mmol) at rt. The reaction mixture was stirred until the red colour of iodine disappeared (3 min). Then TMS-alkyne 130 (258 mg, 0.838 mmol) was added in dimethylacetamide (0.5 cm³) and the reaction was heated to 80 °C. After 3 h, a solution of aryl iodide 116 (150 mg, 0.372 mmol), copper(I) iodide (6.5 mg, 10 mol%) and PdCl₂(dpdpf) (15.2 mg, 5 mol%) in dimethylacetamide (2 cm³) was added and the reaction was stirred overnight (18 h) at 80 °C. The reaction mixture was filtered through celite, the filtrate was diluted with
Experimental

H₂O (30 cm³) and extracted with EtOAc (3 x 30 cm³). The combined organics were dried (MgSO₄) and concentrated *in vacuo*. Purification of the crude solid by column chromatography (hexane:EtOAc, 1:1) gave the product 131 as a colourless solid (153 mg, 82 %). RF (hexane:EtOAc, 1:1) = 0.21; ¹H NMR δ (500 MHz, CDCl₃) 7.08 (4H, m, 4 x ArH), 5.72 (1H, s, NH), 4.34 (4H, m, 2 x CH₂OCOCH₃), 2.61 – 2.52 (4H, m, 2 x CH₂Ar), 2.23 – 2.14 (4H, m, CH₂CC + ArCH₂CH₂C), 2.07 (6H, s, 2 x OCOCH₃), 1.94 (3H, s, NHCOC₂H₅), 1.58 (2H, p, J = 7.8 Hz, ArCH₂CH₂), 1.50 (2H, p, J = 7.1 Hz, CH₂CH₂CC), 1.45 – 1.28 (4H, m, 2 x CH₂), 0.13 (9H, s, 3 x CH₃); ¹³C NMR δ (126 MHz, CDCl₃) 170.87 (C), 170.13 (C), 140.64 (C), 138.56 (C), 128.62 (2 x CH), 128.31 (2 x CH), 107.74 (C), 84.42 (C), 64.70 (2 x CH₂), 58.33 (C), 35.50 (CH₂), 33.80 (CH₂), 31.46 (CH₂), 29.33 (CH₂), 28.79 (CH₂), 28.69 (CH₂), 28.63 (CH₂), 24.22 (CH₃), 20.93 (2 x CH₃), 19.93 (CH₃), 0.29 (3 x CH₃); m/z (ESI+, MeCN) 1025 ([2M+Na]⁺, 2%), 524 ([M+Na]⁺, 100), 524 ([M+H]⁺, 5); HRMS (ESI+, MeCN) [M+Na]⁺ found 524.2809, C₂₈H₄₃NO₅SiNa requires 524.2803.

2-Amino-2-[2-[4-(oct-7-yn-1-yl)phenyl]ethyl]propane-1,3-diol 100

To TMS-alkyne 131 (70 mg, 0.137 mmol) in wet MeOH (10 cm³) was added K₂CO₃ (30 mg, 0.209 mmol) and stirred at rt. After 2 h, another portion of K₂CO₃ (60 mg, 0.420 mmol) was added and stirred overnight at rt. The reaction mixture was concentrated *in vacuo* and purified by column chromatography to give the product 100 as a colourless solid (42 mg, quant.).

5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-{3-[4-(6-{4-[3-amino-4-hydroxy-3-(hydroxymethyl)butyl]phenyl}hexyl)-1H-1,2,3-triazol-1-yl]propyl}pentanamide 137
Experimental

General procedure B was followed with biotin azide 75 (10 mg, 0.031 mmol); fingolimod alkyne 100 (9.3 mg, 0.031 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH₃, 9:1:1) to give the product 137 as a colourless solid (15 mg, 75%).

**1H NMR** δ (601 MHz, CD₃OD) 7.75 (1H, s, CH (triazole)), 7.13 (2H, d, J = 8.0 Hz, 2 x ArH), 7.09 (2H, d, J = 8.0 Hz, 2 x ArH), 4.48 (2H, dd, J = 7.8, 5.0 Hz, NHCH₂CH₂), 4.39 (2H, t, J = 7.0 Hz, CH₂N), 4.30 (2H, dd, J = 7.8, 4.5 Hz, ArH), 3.72 – 3.66 (4H, m, 2 x CH₂OH), 3.23 – 3.17 (3H, m, NHCHCH + CH₂NH), 2.92 (1H, dd, J = 12.7, 5.0 Hz, NHCHCH₃H₃), 2.69 (1H, d, J = 12.7 Hz, NHCHCH₃H₃), 2.67 (2H, t, J = 7.7 Hz, CHCH₂), 2.66 – 2.60 (2H, m, ArCH₃), 2.56 (2H, t, J = 7.6 Hz, CH₂Ar), 2.21 (2H, t, J = 7.4 Hz, CH₂CO), 2.08 (2H, p, J = 6.9 Hz, CH₂CH₂N), 1.98 – 1.89 (2H, m, ArCH₂CH₂), 1.78 – 1.54 (10H, m, 5 x CH₂), 1.48 – 1.32 (4H, m, 2 x CH₂); **13C NMR** δ (151 MHz, CD₃OD) 176.27 (C), 149.22 (C), 141.83 (C), 139.57 (C), 129.62 (2 x CH), 129.17 (2 x CH), 123.37 (CH), 63.36 (CH), 62.55 (2 x CH₂), 62.06 (C), 61.64 (CH), 56.98 (CH), 48.91 (CH₂), 41.03 (CH₂), 37.43 (CH₂), 36.76 (CH₂), 36.37 (CH₂), 34.75 (CH₂), 32.57 (CH₂), 31.13 (CH₂), 30.47 (CH₂), 29.99 (CH₂), 29.88 (CH₂), 29.77 (CH₂), 29.67 (CH₂), 29.48 (CH₂), 26.79 (CH₂), 26.22 (CH₂); m/z (ESI+, MeCN) 652 ([M+Na]⁺, 40%), 630 ([M+H]⁺, 100); **HRMS** (ESI+, MeCN) [M+H]⁺ found 630.3784, C₃₂H₅₂N₇O₄S requires 630.3796.

(2S)-N-(2-{5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido}ethyl)-6-4-(6-{4-amino-4-hydroxy-3-(hydroxymethyl)butyl}phenyl)hexyl)-1H-1,2,3-triazol-1-yl)-2-[3-(3-methyl-3H-diazirin-3-yl)propanamido]hexanamide 138
**General procedure B** was followed with biotin diazirine azide 77 (10 mg, 0.018 mmol); fingolimod alkyne 100 (5.5 mg, 0.018 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH₃, 9:1:1) to give the product 138 as a colourless solid (11 mg, 73%).

**¹H NMR** δ (601 MHz, CD₃OD) 7.74 (1H, s, CH (triazole)), 7.15 – 7.06 (4H, m, 4 x ArH), 4.65 (1H, d, J = 12.1 Hz, OH), 4.57 (1H, d, J = 12.1 Hz, OH), 4.49 (2H, dd, J = 7.8, 4.9 Hz, NHCHCH₂), 4.38 (2H, t, J = 7.0 Hz, CH₂N), 4.31 (1H, dd, J = 7.9, 4.5 Hz, NHCHCH₂), 4.21 (1H, dd, J = 9.1, 5.2 Hz, COCH), 3.69 (2H, t CH₂OH), 3.68 (2H, m, CH₂NH), 3.37 (2H, m, CH₂NH + NHCHCH₂), 2.92 (1H, dd, J = 12.8, 5.0 Hz, NHCHCH₃H), 2.70 (2H, d, J = 13.0 Hz, NHCHCH₃H), 2.70 – 2.64 (2H, m, CHCCCH), 2.65 – 2.60 (2H, m, ArCH₂), 2.57 (4H, t, J = 7.6 Hz, CH₂Ar), 2.26 – 2.10 (4H, m, 2 x CH₂CO), 2.06 – 1.99 (2H, m, CHCCCH₂CH₂), 1.99 – 1.87 (4H, m, 2 x CH₂), 1.85 – 1.56 (1H, m, 6 x CH₂), 1.66 (2H, t, J = 7.7 Hz, CH₂), 1.51 – 1.29 (6H, m, 3 x CH₂), 1.01 (3H, s, CH₃); **¹³C NMR** δ (126 MHz, CD₃OD) 176.45 (C), 174.71 (C), 174.66 (C), 166.15 (C), 149.08 (C), 141.86 (C), 139.56 (C), 129.64 (2 x CH), 129.17 (2 x CH), 123.33 (CH), 63.21 (CH), 62.56 (2 x CH₂), 62.06 (C), 61.70 (CH), 56.92 (CH), 54.77 (CH), 51.10 (CH₂), 41.03 (CH₂), 40.33 (CH₂), 39.98 (CH₂), 36.77 (CH₂), 36.39 (CH₂), 34.77 (CH₂), 32.59 (CH₂), 32.26 (CH₂), 31.21 (CH₂), 31.05 (CH₂), 30.71 (CH₂), 30.46 (CH₂), 30.04 (CH₂), 29.91 (CH₂), 29.69 (CH₂), 29.59 (CH₂), 29.45 (CH₂), 26.79 (CH₂), 26.41 (C), 26.17 (CH₂), 23.88 (CH₂), 19.79 (CH₃); **m/z** (ESI+, MeCN) 854 ([M+H]⁺, 100%); **HRMS** (ESI+, MeCN) [M+H]⁺ found 854.5078, C₄₂H₆₈N₁₁O₆S requires 854.5069.
Experimental

(2S)-N-(2-[5-{(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl}pentanamido]ethyl)-6-[4-{6-[4-[3-amino-4-hydroxy-3-(hydroxymethyl)butyl]phenyl]hexyl]-1H-1,2,3-triazol-1-yl]-2-[(4-benzoylphenyl)formamido]hexanamide 139

General procedure B was followed with biotin benzophenone azide 78 (10 mg, 0.015 mmol); fingolimod alkyne 100 (4.7 mg, 0.015 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH₃, 9:1:1) to give the product 139 as a colourless solid (11 mg, 78%).

¹H NMR δ (601 MHz, CD₃OD) 8.00 (2H, d, J = 8.4 Hz, 2 x ArH (benzophenone, R1)), 7.84 (2H, d, J = 8.4 Hz, 2 x ArH (benzophenone, R1)), 7.80 (2H, d, J = 7.1 Hz, 2 x ArH (benzophenone, R2)), 7.70 (1H, s, CH, triazole), 7.67 (1H, t, J = 7.5 Hz, ArH (benzophenone, R2)), 7.55 (2H, t, J = 7.8 Hz, 2 x ArH (benzophenone, R2)), 7.12 (2H, d, J = 8.0 Hz, 2 x ArH), 7.07 (2H, d, J = 8.0 Hz, 2 x ArH), 4.50 – 4.44 (2H, m, NHCHCH₂ + COCH), 4.40 (1H, t, J = 6.8 Hz, CH₂N), 4.26 (1H, dd, J = 7.8, 4.6 Hz, NHCHCH), 3.71 – 3.63 (4H, m, 2 x CH₂OH), 3.5 – 3.37 (2H, m, CH₂NH), 3.24 – 3.15 (2H, m, CH₂NH), 3.14 (1H, td, J = 7.3, 4.4 Hz, NHCHCH), 2.88 (1H, dd, J = 12.8, 5.0 Hz, NHCHCH)₂Hb), 2.70 – 2.58 (5H, m, NHCHCH)₂Hb + ArCH₂ + CHCCH₂), 2.56 – 2.50 (2H, m, CH₂Ar), 2.23 – 2.09 (2H, m, CH₂CO), 2.03 – 1.80 (6H, m, 3 x CH₂), 1.71 – 1.25 (10H, m, 5 x CH₂); ¹³C NMR δ (151 MHz, CD₃OD) 197.64 (C), 176.48 (C), 174.79 (C), 169.36 (C), 166.15 (C), 149.24 (C), 141.78 (C), 141.55 (C), 139.68 (C),
Experimental

138.51 (C), 138.36 (C), 134.19 (CH), 131.10 (2 x CH), 130.99 (2 x CH), 129.67 (2 x CH), 129.61 (2 x CH), 129.17 (2 x CH), 128.81 (2 x CH), 123.12 (CH), 62.99 (CH), 62.85 (2 x CH₂), 61.73 (CH), 61.68 (C), 56.83 (CH), 55.62 (CH), 50.93 (CH₂), 49.57 (CH₂), 40.99 (CH₂), 40.32 (CH₂), 40.16 (CH₂), 36.79 (CH₂), 36.37 (CH₂), 34.98 (CH₂), 32.57 (CH₂), 32.18 (CH₂), 30.72 (CH₂), 30.49 (CH₂), 30.04 (CH₂), 29.89 (CH₂), 29.71 (CH₂), 29.44 (CH₂), 29.41 (CH₂), 26.85 (CH₂), 26.23 (CH₂), 24.08 (CH₂); \textit{m/z} (ESI+, MeCN) 952 ([M+H]^+ 100%); \textbf{HRMS} (ESI+, MeCN) [M+H]^+ found 952.5109, C₅₁H₇₀N₉O₇S requires 952.5113.

5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-2-[(4-[E]-2-(4-3-4-(6-[4-amino-4-hydroxy-3-(hydroxymethyl)butyl]phenyl)hexyl)-1H-1,2,3-triazol-1-yl|propoxy|phenyl]diazen-1-yl|phenyl|formamido)ethyl|pentanamide 140

\begin{center}
\includegraphics[width=0.8\textwidth]{5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]-N-2-[(4-[E]-2-(4-3-4-(6-[4-amino-4-hydroxy-3-(hydroxymethyl)butyl]phenyl)hexyl)-1H-1,2,3-triazol-1-yl|propoxy|phenyl]diazen-1-yl|phenyl|formamido)ethyl|pentanamide.png}
\end{center}

\textbf{General procedure B} was followed with biotin linker azide 54 (10 mg, 0.017 mmol); fingolimod alkyne 100 (5.1 mg, 0.017 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH₃, 18:2:1) to give the product 140 as an orange solid (12.5 mg, 82%). \textbf{1H NMR} δ (500 MHz, (CD₃)₂SO) 8.66 (1H, t, \textit{J} = 5.5 Hz, NH), 8.03 (2H, d, \textit{J} = 8.5 Hz, 2 x ArH), 7.96 (1H, t, \textit{J} = 5.4 Hz, NH), 7.92 (2H, d, \textit{J} = 9.0 Hz, 2 x ArH), 7.89 (2H, d, \textit{J} = 8.5 Hz, 2 x ArH), 7.88 (1H, s, CH, triazole), 7.12 (2H, d, \textit{J} = 9.0 Hz, 2 x ArH) 7.08 (4H, br s, 4 x ArH), 6.40 (1H, s, NH), 6.33 (1H, s, NH), 5.31 (2H, s, NH₂), 4.50 (1H, t, \textit{J} = 6.9 Hz, CH₂N), 4.27 (1H, dd, \textit{J} = 7.7, 5.1 Hz, NHCHCH₂), 4.15 – 4.04 (3H, m, NHCHCH + CH₂O), 3.50 (4H, s, 2 x CH₂OH), 3.36 – 3.22 (4H, m, 2 x CH₂NH), 3.04 (1H, ddd, \textit{J} = 8.5, 6.2, 4.4 Hz, NHCHCH), 2.79 (1H, dd, \textit{J} = 12.5, 5.2 Hz, NHCHCH₂H₅), 2.66 –
2.51 (5H, m, NHCHCH₉H + ArCH₂ + CHCCH₂), 2.31 (2H, p, J = 6.8 Hz, CH₂CH₂N), 2.08 (2H, t, J = 7.4 Hz, CH₂CO), 1.80 – 1.69 (2H, m, CH₂CH₂Ar), 1.65 – 1.39 (8H, m, 4 x CH₂), 1.37 – 1.04 (4H, m, 2 x CH₂); ¹³C NMR δ (126 MHz, (CD₃)₂SO) 172.39 (C), 165.58 (C), 162.65 (C), 161.49 (C), 153.37 (C), 146.89 (C), 146.27 (C), 139.71 (C), 138.84 (C), 136.00 (C), 128.45 (2 x CH), 128.22 (2 x CH), 127.98 (2 x CH), 124.83 (2 x CH), 121.97 (2 x CH), 121.89 (CH), 115.15 (2 x CH), 65.11 (CH₂), 61.33 (2 x CH₂), 60.97 (CH), 59.81 (C), 59.16 (CH), 55.35 (CH), 46.21 (CH₂), 39.80 (CH₂), 38.16 (CH₂), 35.24 (CH₂), 34.68 (CH₂), 33.50 (CH₂), 30.93 (CH₂), 29.36 (CH₂), 28.98 (CH₂), 28.90 (CH₂), 28.67 (CH₂), 28.38 (CH₂), 28.36 (CH₂), 28.14 (CH₂), 28.01 (CH₂), 27.95 (CH₂), 25.22 (CH₂), 24.98 (CH₂); m/z (ESI+, MeCN) 919 ([M+Na]⁺, 15%), 897 ([M+H]⁺, 100), 449 ([M+2H]²⁺, 20); HRMS (ESI+, MeCN) [M+H]⁺ found 897.4848, C₄₇H₆₅N₁₀O₆S requires 897.4804.

(2S)-N-(3-{4-[(E)-2-{4-{5-[(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido}ethyl]carbamoyl}phenyl)diazen-1-yl[phenoxy]propyl)-6-{6-{4-{3-amino-4-hydroxy-3-(hydroxymethyl)butyl}phenyl}hexyl]-1H-1,2,3-triazol-1-yl]-2-{3-(3-methyl-3H-diazirin-3-yl)propanamido}hexanamide 141
**General procedure B** was followed with biotin linker diazirine azide 70 (10 mg, 0.012 mmol); fingolimod alkyne 100 (3.7 mg, 0.012 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH₃, 18:2:1) to give the product 141 as an orange solid (10 mg, 72%). \( R_f \) (DCM:MeOH:NH₃, 18:2:1) = 0.41; \(^1\)H NMR \( \delta \) (601 MHz, (CD₃)₂SO) 8.03 (2H, d, \( J = 8.5 \) Hz, 2 x ArH), 7.92 (2H, d, \( J = 8.9 \) Hz, 2 x ArH), 7.89 (2H, d, \( J = 8.5 \) Hz, 2 x ArH), 7.76 (1H, s, CH, triazole), 7.13 (2H, d, \( J = 8.9 \) Hz, 2 x ArH), 7.08 (4H, br s, 4 x ArH), 6.40 (1H, s, NH), 6.34 (1H, s, NH), 4.28 (1H, dd, \( J = 7.8, 5.2 \) Hz, NHCHCH₂), 4.24 (1H, t, \( J = 7.0 \) Hz, CH₂N), 4.17 (1H, td, \( J = 8.4, 5.5 \) Hz, COCH(NH), 4.13 – 4.06 (3H, m, NHCHCH + OCH₂), 3.49 (4H, s, 2 x CH₂OH), 3.28 – 3.19 (4H, m, 2 x CH₂NH), 3.04 (1H, ddd, \( J = 8.5, 6.2, 4.4 \) Hz, NHCHCH), 2.79 (1H, dd, \( J = 12.4, 5.1 \) Hz, CH₃H₂S), 2.59 – 2.51 (5H, m, CH₃H₂S + 2 x CH₂), 2.08 (2H, t, \( J = 7.4 \) Hz, CH₂CO), 2.02 (2H, t, \( J = 8.0 \) Hz, CH₂), 1.91 – 1.81 (2H, m, CH₂), 1.81 – 1.70 (4H, m, 2 x CH₂), 1.67 – 1.39 (12H, m, 6 x CH₂), 1.36 – 1.03 (12H, m, 6 x CH₂), 0.96 (3H, s, CCH₃); \(^{13}\)C NMR \( \delta \) (126 MHz, (CD₃)₂SO) 172.42 (C), 171.54 (C), 170.72 (C), 165.61 (C), 162.67 (C), 161.77 (C), 153.40 (C), 146.76 (C), 146.15 (C), 139.73 (C), 138.85 (C), 135.96 (C), 128.44 (2 x CH), 128.23 (2 x CH), 127.98 (2 x CH), 124.84 (2 x CH), 121.97 (2 x CH), 121.46 (CH), 115.13 (2 x CH), 65.75 (CH₂), 61.40 (2 x CH₂), 60.98 (CH), 59.73 (C) 59.17 (CH), 55.36 (CH), 52.30 (CH), 48.96 (CH₂), 39.82 (CH₂), 39.41 (CH₂), 38.17 (CH₂), 35.34 (CH₂), 35.25 (CH₂), 34.70 (CH₂), 33.55 (CH₂), 31.43 (CH₂), 30.95 (CH₂), 29.80 (CH₂), 29.57 (CH₂), 29.32 (CH₂), 28.95 (CH₂), 28.67 (CH₂), 28.45...
(CH,) 28.40 (CH,) 28.15 (CH,) 28.03 (CH,) 27.97 (CH,) 25.77 (C) 25.24 (CH,) 25.03 (CH,) 22.29 (CH,) 19.25 (CH,); m/z (ESI+, MeCN) 1157 ([M+Na]+, 40%), 1135 ([M+H]+, 100); HRMS (ESI+, MeCN) [M+H]+ found 1135.6261, C58H83N14O8S requires 1135.6234.

(2S)-N-(3-{4-{(E)-2-{4-{2-{5-{(3aS,4S,6aR)-2-oxo-hexahydro-1H-thieno[3,4-d]imidazolidin-4-yl]pentanamido}ethyl}carbamoyl}phenyl}diazen-1-yl[phenoxy]propyl)-6-{4-{6-{4-{3-amino-4-hydroxy-3-(hydroxymethyl)butyl}phenyl}hexyl]-1H-1,2,3-triazol-1-yl]-2-[(4-benzyloxyphe nyl)formamido]hexanamide 142

General procedure B was followed with biotin linker benzophenone azide 71 (10 mg, 0.011 mmol); fingolimod alkyne 100 (3.3 mg, 0.011 mmol). The reaction mixture was purified using column chromatography (5-10% MeOH in DCM; then DCM:MeOH:NH3, 18:2:1) to give the product 142 as an orange solid (9 mg, 71%). Rf (DCM:MeOH:NH3, 18:2:1) = 0.31; 1H NMR (601 MHz, (CD3)2SO) 8.04 (2H, d, J = 8.3 Hz, 2 x ArH), 8.02 (4H, d, J = 8.7 Hz, 2 x ArH), 7.89 (2H, d, J = 8.9 Hz, 2 x ArH), 7.78 (2H, d, J = 8.4 Hz, 2 x ArH), 7.74 (2H, d, J = 6.9 Hz, 2 x ArH), 7.71 – 7.68 (1H, m, ArH), 7.57 (2H, m, 2 x ArH), 7.12 (2H, d, J = 9.0 Hz, 2 x ArH), 7.08 (4H, s, 4 x ArH), 6.40 (1H, s,
N\textsubscript{H}), 6.33 (1H, s, N\textsubscript{H}), 4.44 – 4.37 (1H, m, COCHNH), 4.31 – 4.25 (3H, m, NHCH\textsubscript{2}CH + CH\textsubscript{2}N), 4.15 – 4.05 (3H, m, NHCHCH + OCH\textsubscript{2}), 3.51 (4H, s, 2 x CH\textsubscript{2}OH), 3.27 – 3.23 (4H, m, 2 x CH\textsubscript{2}NH), 3.04 (1H, ddd, $J = 8.5, 6.2, 4.4$ Hz, NHC\textsubscript{H}C\textsubscript{H}CO), 2.79 (1H, dd, $J = 12.4, 5.1$ Hz, CH\textsubscript{2}H\textsubscript{3}S), 2.59 – 2.52 (5H, m, CH\textsubscript{3}H\textsubscript{5}S + 2 x CH\textsubscript{2}), 2.08 (3H, t, $J = 7.4$ Hz, CH\textsubscript{2}CO), 1.95 – 1.24 (28H, m, 14 x CH\textsubscript{2}). $^{13}$C NMR (151 MHz, (CD\textsubscript{3})\textsubscript{2}SO) $\delta$ 195.85 (C), 172.88 (C), 172.04 (C), 166.11 (C), 163.14 (C), 162.24 (C), 153.85 (C), 147.22 (C), 146.61 (C), 140.24 (C), 139.66 (C), 139.20 (C), 137.88 (C), 137.13 (C), 136.42 (C), 133.49 (CH), 130.14 (2 x CH), 129.78 (2 x CH), 129.13 (2 x CH), 128.90 (2 x CH), 128.71 (2 x CH), 128.45 (2 x CH), 128.21 (2 x CH), 125.29 (2 x CH), 122.43 (2 x CH), 121.98 (CH), 115.60 (2 x CH), 66.26 (CH\textsubscript{2}), 61.58 (2 x CH\textsubscript{2}), 61.45 (CH), 60.54 (C), 59.64 (CH), 55.84 (CH), 53.92 (CH), 49.41 (CH\textsubscript{2}), 38.64 (CH\textsubscript{2}), 35.93 (CH\textsubscript{2}), 35.73 (CH\textsubscript{2}), 35.16 (CH\textsubscript{2}), 31.41 (CH\textsubscript{2}), 31.38 (CH\textsubscript{2}), 29.79 (CH\textsubscript{2}), 29.41 (CH\textsubscript{2}), 29.16 (CH\textsubscript{2}), 28.91 (CH\textsubscript{2}), 28.86 (CH\textsubscript{2}), 28.63 (CH\textsubscript{2}), 28.50 (CH\textsubscript{2}), 28.40 (CH\textsubscript{2}), 25.71 (CH\textsubscript{2}), 25.47 (CH\textsubscript{2}), 23.12 (CH\textsubscript{2}); m/z (ESI+, MeCN) 1233 ([M+H]\textsuperscript{+}, 100%); HRMS (ESI+, MeCN) [M+H]\textsuperscript{+} found 1233.6277, C\textsubscript{67}H\textsubscript{85}N\textsubscript{12}O\textsubscript{9}S requires 1233.6278.
5.5 Experimental for Chapter 4

5.5.1 Anisomycin

Ribosome purification

All solutions and cell fractions were kept at 4 °C unless otherwise stated. Four stock solutions were prepared; Buffer A (250 mM sucrose, 250 mM KCl, 5 mM MgCl₂, 50 mM HEPES pH 7.4), Buffer B (250 mM sucrose 0.5 M KCl, 5 mM MgCl₂, 50 mM HEPES pH 7.4), Buffer C (25 mM KCl, 5 mM MgCl₂, 50 mM HEPES pH 7.4) and Sucrose Cushion (1 M sucrose, 0.5 M KCl, 5 mM MgCl₂, 50 mM HEPES pH 7.4).

Harvested cells (MDA-MB-468, 15 x 10⁷ cells by Trypan Blue count) were removed from the freezer, placed in ice and resuspended gently by pipetting the cellular pellet with cold buffer A (3 x 1 cm³) in three sequential steps with homogenisation between additions of buffer A. The detergent NP-40 (0.28 cm³) was added to the cellular suspension to perform cell lysis, and was incubated on ice (15 min). The solution was homogenised by gently pipetting at the beginning of the incubation and 5 min following the incubation. In the cell lysis step, the cytoplasm detaches from the nuclei, NP-40 is a non-ionic detergent that facilitates cell lysis by inducing weakness of the plasma membrane. A small aliquot (100 µL) of the whole cell lysate was retained for SDS-PAGE analysis. The cell lysate was centrifuged (750 x g, 4 °C, 10 min) to pellet the nuclei. The supernatant contained the cytoplasmic ribosomes and was retained while the pellet containing nuclei was kept for further SDS-PAGE analysis. The cytoplasmic supernatant was centrifuged (12500 x g, 4 °C, 10 min), the pellet formed contained mitochondria, and was retained for SDS-PAGE analysis. The supernatant is the post mitochondrial fraction (PMT) which contains ribosome, and a small aliquot of the supernatant was retained for SDS-PAGE analysis. The PMT volume was accurately measures (PMT_VOL = 2.8 cm³) and KCl (0.2 cm³, 4 M) was added slowly to the PMT fraction to give a final concentration of 0.5 M. Sucrose cushion (1 cm³) was added to each of the 4 polycarbonate tubes (5.1 cm³), and the KCl adjusted PMT fraction (0.75 cm³) was slowly added to each tube on top of the sucrose cushion. The tubes were centrifuged (250000 x g, 4 °C, 2 hr) and the supernatant (post-ribosomal
fraction) was discarded, and the dense ribosomal pellets were washed quickly with cold deionised water (0.2 cm$^3$). The ribosome pellets were resuspended with buffer C (3 x 0.1 cm$^3$), after each addition of buffer the pellets were homogenised gently to obtain a ribosomal suspension. A small aliquot of suspended ribosomes was kept for SDS-PAGE analysis, and the rest of the ribosomes obtained were used in the competition assay.

**SDS-PAGE**

The gel was run using 12% SDS-PAGE gel and was prepared using as follows: deionised H$_2$O (3.29 cm$^3$), 30% acrylamide mix (4 cm$^3$), 1.5M Tris.HCl pH 8.8 (2.5 cm$^3$), 10% SDS (100 µL), 10% ammonium persulfate (100 µL), and TEMED (10 µL).  

The gel was left to set in 1.0 mm plates with n-propanol (1 cm$^3$) pipetted on top of the phase boundary to create a straight edge. After setting, the n-propanol was removed and the stacking gel was pipetted on top, which was prepared as follows: deionised H$_2$O (2.74 cm$^3$), 30% acrylamide mix (0.68 cm$^3$), 1.0M Tris.HCl pH 6.8 (0.5 cm$^3$), 10% SDS (40 µL), 10% ammonium persulfate (40 µL), and TEMED (4 µL). The gel comb was inserted into the top of the plates, and left in the gel until the gel was set.

The SDS loading buffer used was Laemmli sample buffer, the 2X buffer contains as follows: SDS (4%), glycerol (20%), 2-mercaptoethanol (10%), bromphenol blue (0.004%) and Tris.HCl pH 6.8 (0.125 M). The Laemmli/DTT mix buffer was prepared using 2X Laemmli (0.9 cm$^3$) and 1 M DTT (0.1 cm$^3$). The 5 samples used in the gel were taken from the ribosomal purification; whole cell lysate, post-nuclear fraction, mitochondrial fraction, post mitochondrial fraction and ribosomal fraction. A sample of each fraction (50 µL) was incubated with Laemmli/DTT mix (50 µL) at 95 °C for 5 min.

In order to carry out the SDS-PAGE, a Bradford assay was carried out. This colorimetric protein assay is a spectroscopic analytical procedure used to analyse the protein content in a sample based on the absorbance shift of the Bradford reagent dye. The protein-bound form of the dye has an absorption spectrum maximum at 595 nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and
thus the concentration of protein present in the sample. Calculation of the total protein concentration in a sample allows appropriate dilution factors to be applied in the SDS-PAGE experiment – so that all the protein contents are the same. From the Bradford assay, the protein content of each fraction was found as annotated below in Figure 5.1.

<table>
<thead>
<tr>
<th>Fraction from purification</th>
<th>Protein content (µg)</th>
<th>Protein content in Laemmli (µg/µl)</th>
<th>Dilution</th>
<th>Vol IX Laemmli added (µl)</th>
<th>Protein content in 30 µl sample (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cell lysate</td>
<td>5.16</td>
<td>2.58</td>
<td>1:10</td>
<td>90</td>
<td>7.5</td>
</tr>
<tr>
<td>Post-nuclear</td>
<td>5.92</td>
<td>2.96</td>
<td>1:12</td>
<td>110</td>
<td>7.5</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>5.97</td>
<td>2.98</td>
<td>1:12</td>
<td>110</td>
<td>7.5</td>
</tr>
<tr>
<td>Post-mitochondrial</td>
<td>6.61</td>
<td>3.31</td>
<td>1:13.2</td>
<td>120</td>
<td>7.5</td>
</tr>
<tr>
<td>Ribosomal</td>
<td>0.52</td>
<td>0.26</td>
<td>n/a</td>
<td>n/a</td>
<td>7.5</td>
</tr>
</tbody>
</table>

**Figure 5.1** – A table showing the sample preparation for SDS-PAGE using Bradford assay.

The gel was run using the diluted samples (30 µL) in each lane A-E (Figure 4.2), and the marker (BIORAD Precision Plus protein prestained ladder, 15 µL) was pipetted into lane M.

**Figure 5.2** – SDS page gel for ribosomal preparation. (M) contains the BioRad molecular marker; (A) is from the whole cell lysate; (B) is the post-nuclear fraction; (C) is the mitochondrial fraction; (D) is the post-mitochondrial fraction; (E) is the ribosomal fraction.
The gel was stained using Coomassie blue dye, excess dye was removed using destain (40% methanol, 5% acetic acid in H$_2$O) and then rinsed using water. The proteins were transferred from the gel onto a PVDF (polyvinylidene difluoride) which was carried out by electroblotting. After the transfer, the membrane was blocked using 3% milk solution (1.5 g milk powder in 50 cm$^3$ H$_2$O). The protein in the dilute milk solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives.

![Figure 5.3](image)

**Figure 5.3** – Fluorescence of ribosomal protein by immunoblotting.

The primary antibody (S3; FL-243) was added to the membrane (1 h), the excess removed and the membrane was washed (3 x 10 min) using TBST (Tris-buffered saline and Tween 20). The secondary antibody was then added to the membrane (1 h), the excess was removed and the membrane was washed (3 x 10 min) using TBST (Tris-buffered saline and Tween 20). The secondary antibody was linked to horseradish peroxidase (HRP) in order to create fluorescence in proportion to the amount of ribosomal protein. To measure the fluorescence, HRP substrate peroxide solution (3 cm$^3$) and HRP substrate luminol reagent (3 cm$^3$) were mixed together and then added to the membrane. The fluorescence was visualised using a photosensor (CCD camera), as seen in Figure 5.3.
Fluorescence Assay

Ribosomes in buffer C (50 µL) obtained from the purification were incubated (4 °C, 3 h) with the controls (ANS, 75, 53, 5% BSA in buffer C, 3 x 10^-5 M) and the biotin-anchored affinity tags (89, 92, 3 x 10^-5 M), with anisomycin (3 x 10^-4 M), as described in Figure 5.4.

<table>
<thead>
<tr>
<th>Ribosomes</th>
<th>Biotin-Linker</th>
<th>Biotin-linker-ANS</th>
<th>Anisomycin</th>
<th>SA-HRP</th>
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</table>

Figure 5.4 – A competition assay layout, where (+) means it was added, and (-) means it was omitted.

After incubation each sample (45 µL) was ultracentrifuged (250000 x g, 4°C, 1 h) through a sucrose cushion (300 µL), and the pellets were resuspended in buffer C (100 µL). Serial dilutions of the samples (1:3) were carried out using buffer C and each sample dilution (20 µL) was placed in a plate with a solution of the QuantaBlu stable peroxide and the QuantaBlu substrate (180 µL, 1:10). The fluorescence was then recorded.
5.5.2 Fingolimod

Preliminary affinity studies

Materials and methods

SDS-PAGE materials and NeutrAvidin agarose resin were purchased from Invitrogen (Thermo Scientific); Novex NuPage® 4-12% Bis-Tris gels were run according to manufacturer’s descriptions using 2x SDS loading buffer and 1x MES running buffer from Invitrogen. Molecular weight markers were SeeBlu® pre-stained protein standards from Invitrogen (Thermo Scientific). Phosphate buffered saline (PBS) was prepared by dissolving PBS tablets from Sigma Aldrich. HeLa lysate was prepared off-site at the Edinburgh Cancer Research Centre by Ifigeneia Stavrou using RIPA cell lysis buffer, and supplied with an estimated protein content (25 mg/mL) by a Nanodrop™ spectrophotometer.

2x SDS loading buffer – 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, Tris-HCl (0.125 M), pH 6.8.

1x MES buffer – MES (50 mM), Tris Base (50 mM), 0.1% SDS, EDTA (1 mM), pH 7.3.

1x PBS – One tablet dissolved in deionized water (200 cm³): phosphate buffer (0.01 M), potassium chloride (0.0027 M) and sodium chloride (0.137 M), pH 7.4, at 25 °C.

RIPA buffer – NaCl (150 mM), 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, Tris-HCl (50 mM), pH 8.0, protease inhibitors.

NeutrAvidin® bead activation

To NeutrAvidin® agarose resin (200 µL, of which 100 µL was beads) in an Eppendorf was added PBS buffer (200 µL), the mixture was agitated, centrifuged (17 x g, 5 min), and the supernatant was decanted. The beads were washed a further 3 times with PBS buffer (200 µL). To the pelleted beads was added PBS buffer (200 µL) and affinity probe (200 µL, 5 mg/mL in 1:1 DMSO:H₂O), and the mixture was agitated overnight.
(18 h, 1000 rpm, 4 °C). The activated resin was then centrifuged (17 x g, 5 min), the supernatant was removed, and the pelleted beads were washed with PBS buffer (4 x 400 µL).

**Affinity experiment**

To the pelleted activated beads was added PBS buffer (100 µL), HeLa cell lysate (100 µL) and DMSO (2 µL), and the mixture was agitated overnight (18 h, 1000 rpm, 4 °C). The mixture was then centrifuged (17 x g, 5 min), the supernatant was removed and the pelleted beads were washed with PBS buffer (4 x 400 µL).

To the pelleted beads was added 2x SDS loading buffer (50 µL) and heated in a water bath (5 min, 100 °C). After heating the mixture was centrifuged (17 x g, 5 min), and the supernatants were analysed by SDS-PAGE. The gels were visualised using

**Western blotting**

**Materials and methods**

Image analysis was carried out using Amersham Biosciences Typhoon 9400 Variable Mode Imager (600 V, 11 min run time). GE Healthcare Amersham ECL Prime Western blot Detection Reagents were used for detection of antibody. Anti-biotin, HRP-linker antibody was purchased from Cell Signalling Technology, and was used according to manufacturer’s description. Nitrocellulose membrane used was GE Healthcare Amersham Hybond ECL. Electroblotting was carried out using the Bio-Rad Trans-Blot® SD Semi-Dry Transfer Cell and used according to manufacturer’s instructions.

Bjerrum Schafer-Nielsen transfer buffer – Tris base (48 mM), glycine (39 mM), 20% methanol, pH 9.2. 1x PBS-T – 0.1% Tween-20 in 1x PBS. Blocking buffer - 1X PBST with 5% (w/v) nonfat dry milk powder.
Experimental

Protein transfer

The proteins separated by gel electrophoresis were transferred onto a nitrocellulose membrane using electroblotting. Prior to transfer, the membrane, cut to the required size, was wetted with deionised water and then transferred into transfer buffer to equilibrate for 10 min. The gel was also placed into transfer buffer to equilibrate for 10 min. Extra thick filter paper was cut to the dimensions of the gel and membrane, and placed in transfer buffer to soak for 15-20 min. Electroblotting was carried out and set up according to manufacturer’s descriptions (18 V, 10 mins).

Antibody probing

After the transfer, non-specific binding sites on the membrane were blocked overnight at room temperature using the blocking buffer (50 cm$^3$). The membrane was washed with 1x PBS-T (20 cm$^3$, 30 min; then 5 x 1 min washes). The anti-biotin antibody was diluted to an appropriate dilution in 1 x PBS-T (1:1000), and the membrane was incubated with the antibody for 1 h at room temperature. The membrane was washed again with 1x PBS-T (20 cm$^3$, 5 x 5 min washes), then placed in a plastic folder where detection reagents were added according to manufacturer’s instructions. The membrane was incubated (5 min) and excess reagent was removed. The image analysis was carried out using the Amersham Biosciences imager.
Chapter 6 References

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