Multivalent scaffolds for use as protein surface mimetics

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Intellectual Property and Publication Statement

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 1 is adapted from a review article ‘Metal Complexes as Protein Surface Mimetics’, S. H. Hewitt and A. J. Wilson, Chem. Commun., 2016, 52, 9745-9756. The contributions from the authors were as follows: SHH (the candidate) drafted the review, AJW edited the manuscript into its present form.

Work presented in chapter 2 formed the basis of the research article ‘Protein Surface Mimetics; Understanding How Ruthenium Tris (bipyridines) Interact with Proteins’, S. H. Hewitt, M. H. Filby, E. Hayes, L. Kuhn, A. Kalverda, M. E. Webb and A. J. Wilson, ChemBioChem, 2017, 18, 223–231. The contributions from the author were as follows: SHH (the candidate), MHF, EH and AJW designed the research; SHH synthesised the compounds; MHF aided by MEW performed the PPI inhibition experiments; EH and MHF performed luminescence quenching assays; SHH performed the published luminescence quenching assays; SHH, LK and AK obtained the protein NMR spectra; SHH analysed the NMR spectra; SHH drafted the manuscript and AJW edited the manuscript into its present form.

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Abstract

The development of ligands for protein surfaces to inhibit protein-protein interactions (PPIs) is challenging, as protein surfaces often lack the clefts and pockets associated with traditionally druggable targets like enzyme active sites. One way in which protein surfaces can be targeted is by the use of protein surface mimetics, whereby a multivalent scaffold is functionalised with many binding groups on its periphery in order to achieve high affinity protein recognition. One such scaffold is a ruthenium(II) tris (bipyridine) (Ru(II)(bpy)_3).

The work in this thesis aimed to further develop these Ru(II)(bpy)_3 protein surface mimetics; gaining information as to how they interact with proteins, looking at new ways of achieving high affinity protein surface recognition and the development of new applications for these molecules. In Chapter 2 an indepth study of the binding of two Ru(II)(bpy)_3 complexes to a model protein, cytochrome c, is presented, looking at the thermodynamic and electrostatic contributions to binding as well as using protein NMR to elucidate the binding site. In Chapter 3 the development of dynamic combinatorial chemistry (DCC) scaffolds based on Ru(II)(bpy)_3 complexes and tetraphenyl porphyrins was explored as a potential avenue for new receptor design, enabling the development of biologically compatible DCC systems, prime for protein ligand discovery. Chapter 4 presents another avenue for using the Ru(II)(bpy)_3 complexes; using an array approach to discriminate between different protein.
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<tr>
<td>α-ChT</td>
<td>α-chymotrypsin</td>
</tr>
<tr>
<td>ATP</td>
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<tr>
<td>Bak</td>
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<td>ESI</td>
<td>electro-spray ionisation</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fmoc</td>
<td>9-fluorenymethyl oxycarbonyl</td>
</tr>
<tr>
<td>FP</td>
<td>fluorescence polarisation</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HCTU</td>
<td>N,N,N′,N′-Tetramethyl-O-(6-chloro-1H-benzotriazol-1-yl) uranium hexafluorophosphate</td>
</tr>
<tr>
<td>hDM2</td>
<td>human double minute 2</td>
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<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum correlation</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDA</td>
<td>iminodiacetate</td>
</tr>
<tr>
<td>IR</td>
<td>infrared (spectroscopy)</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>K&lt;sub&gt;a&lt;/sub&gt;</td>
<td>association constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>LCMS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LDA</td>
<td>linear discriminant analysis</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>induced myeloid leukemia cell differentiation protein</td>
</tr>
<tr>
<td>MD</td>
<td>molecular dynamics</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
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<tr>
<td>MWCO</td>
<td>molecular weight cut off</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>p53</td>
<td>(tumour) protein 53</td>
</tr>
<tr>
<td>PAMAM</td>
<td>poly(amidoamine)</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<td>PDB</td>
<td>protein data bank</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PET</td>
<td>photo-induced electron transfer</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>phen</td>
<td>phenanthroline</td>
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<td>PNA</td>
<td>peanut agglutinin</td>
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<tr>
<td>PPI</td>
<td>protein-protein interaction</td>
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<tr>
<td>ppy</td>
<td>phenylpyridinato</td>
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<tr>
<td>PyBOP</td>
<td>benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
<tr>
<td>QDA</td>
<td>quadratic discriminant analysis</td>
</tr>
<tr>
<td>RNAse</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SAR</td>
<td>structure-activity relationship</td>
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<tr>
<td>SAXS</td>
<td>small angle x-ray scattering</td>
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<tr>
<td>SH2</td>
<td>src homology domain 2</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropyl silane</td>
</tr>
<tr>
<td>TLC</td>
<td>thin-layer chromatography</td>
</tr>
<tr>
<td>Tm</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
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1 Introduction

This chapter is adapted from ‘Metal Complexes as Protein Surface Mimetics’, S. H. Hewitt and A. J. Wilson, Chem. Commun., 2016, 52, 9745-9756

1.1 Protein-protein interactions

Protein-protein interactions (PPIs) are ubiquitous within biology, being used for a plethora of different processes, including signal transduction, antibody responses, the formation of complex structures, apoptosis and the control of the cell cycle.\(^1\) As a result of their abundant nature, the modulation (inhibition and stabilisation) of PPIs is of great interest both therapeutically and for the study of biological processes.

Many studies have been performed in order to establish the topology and chemical nature of the protein-protein interface, but these have led to many discrepancies, over generic conclusions such as the hydrophobicity and charge content of the interface.\(^2\) However, from these studies it has been established that there is a tendency for the interface to be mainly hydrophobic, like the protein core, but to contain a relatively larger proportion of charged and polar amino acid residues.\(^2\)

1.1.1 Modulation of protein-protein interactions

![Figure 1.1] Targeting enzymes and PPIs a) Targeting a clearly defined enzyme pocket with a small molecule inhibitor, b) Targeting a PPI over a much larger surface area

The nature of protein interfaces makes targeting PPIs notoriously difficult (Figure 1.1); indeed they have been described as ‘undruggable’, or ‘high-hanging fruit’.\(^3\) In order to
target a PPI, a small molecule that binds to a protein surface is required, yet protein surfaces are often large (~1500 – 3000 Å²) and flat, lacking the clefts and pockets associated with more conventional protein targets, like enzyme active sites or G protein-coupled receptors, making it difficult to discover small molecules capable of protein surface recognition.\(^3\)

### 1.1.2 Conventional methods for development of protein surface ligands

Various different approaches have been used to target PPIs. Some successes having been found with peptides and peptidomimetics,\(^4\) along with fragment based approaches, high-throughput screening and virtual screening.\(^5,6\) However PPIs still remain a difficult target for small molecule ligands.

#### 1.1.2.1 High-throughput screening

![Figure 1.2 Nutlin 3a (1), an inhibitor of the hDM2/p53 PPI discovered by high-throughput screening, a) Molecular structure of Nutlin 3a (1), ii) Protein crystal structure of Nutlin 3a (1) bound to hDM2 (PDB ID: 4HG7)](image)

The screening of large libraries of drug-like molecules, followed by further optimization has yielded the Nutlins as inhibitors of the p53/hDM2 interaction (Figure 1.2),\(^7\) the most potent of which (Nutlin 3 (1), IC\(_{50}\) 0.09 μM) has been shown to inhibit tumourigenesis in human xenograft cells \textit{in vivo}.\(^8\) However these high throughput approaches have yielded few results, potentially as the libraries being screened are comprised of molecules ‘designed’ for more traditional drug targets, like G-coupled receptors and enzyme active sites.\(^3\)

#### 1.1.2.2 Fragments

Fragment based approaches involve the screening of small (~300 Da), relatively polar molecules for binding to proteins which can then be further elaborated by linking together separate fragments or growing from one fragment into a larger more drug-like molecule. Fragment based approaches have been used, by the Fesik group, to develop ABT-263 (2),
an inhibitor of the Bcl\textsubscript{XL}/BAK PPI (Figure 1.3),\textsuperscript{9} a drug candidate which entered phase II clinical trials. However, there have been reported problems with the fragment-based approach as applied to PPIs, as fragments required for the targeting of PPIs, are often large compared to that for the more conventional drug targets.\textsuperscript{10}

**Figure 1.3** ABT-263 (2), an inhibitor of the Bcl\textsubscript{XL}/BAK PPI, discovered by fragment-based screening. a) ABT-263 molecular structure, b) Protein crystal structure of ABT-263 bound to Bcl\textsubscript{XL} (PDB ID: 4LVT)

### 1.1.2.3 Secondary structure mimetics

Much work involves the design of mimics of protein secondary structure, mimicking \(\beta\)-turns,\textsuperscript{11} \(\alpha\)-helices\textsuperscript{12} and \(\beta\)-sheets.\textsuperscript{13} These allow the mimicry of the secondary structural elements of one protein binding partner, delivering a molecule able to bind to the other partner, thus inhibiting the PPI. This has potential as a general approach to small molecule structural design, allowing the development of inhibitors for many PPIs based on similar secondary structural interactions, utilizing a single molecular scaffold.

An example of an \(\alpha\)-helical secondary structure mimic is the use of constrained peptides (Figure 1.4). In this approach a sequence of \(\alpha\)-amino acids is preorganised into a helical conformation by, for example, the addition of a hydrocarbon constraint.\textsuperscript{14} Theoretically, this reduces the entropy loss on binding from that of the unconstrained peptide, potentially increasing the binding affinity,\textsuperscript{15} as there is now no barrier to the formation of the bound helical conformation. The introduction of the constraint also increases the stability of the peptide to proteolysis\textsuperscript{14} and can increase the cell permeability.\textsuperscript{16}
1.2 The surface mimetic approach

The surface mimetic approach utilises a large, often supramolecular, scaffold to project binding groups over a large area of protein surface (Figure 1.5)\textsuperscript{17}–\textsuperscript{19} to achieve high affinity protein binding. This allows for the recognition of large areas of protein surface, with fewer discernable features than can be recognised, for example by a secondary structure mimetic. The binding utilizes multivalency, the interaction of multiple binding groups located around a central scaffold on a host molecule (surface mimetic), towards multiple recognition sites on a receptor molecule (protein) in order to achieve high affinity binding.\textsuperscript{19} Multivalency is widely used in nature,\textsuperscript{20} where it allows for an increased binding affinity by increasing the number of ligand and receptor sites, for example in signal transduction, cell membrane adherence, and immunological responses.
1.2.1 Non-metallo protein surface mimetics

Many conventional organic supramolecular scaffolds have been developed for binding to protein surfaces. These include calixarenes, porphyrins, anthracenes, cyclodextrins, resorcinarenes and dendrimers.\textsuperscript{19}

1.2.1.1 Calixarenes

![Figure 1.6](image)

Figure 1.6 Calixarene protein surface binders a) GFB-111 (3), a PDGF ligand, b) Protein crystal structure of two p-sulfonatocalix[4]arenes bound to the surface of cyt c (PDB ID: 3TY1)

Calixarenes are cone-like molecules with two distinct edges that can be functionalised with recognition elements for protein surface recognition.\textsuperscript{21} The Hamilton group have synthesised calix[4]arene derivatives which bind to cytochrome (cyt) c, α-chymotrypsin (α-ChT) and platelet-derived growth factor (PDGF), acting as antibody mimics.\textsuperscript{22–25} GFB-111 (3) (Figure 1.6a), a PDGF binder with IC\textsubscript{50} 250 nM, was shown to be functional in vivo (mouse).\textsuperscript{25} Crowley and coworkers, more recently, solved a crystal structure of a p-sulfonatocalix[4]arene bound to cyt c at three different sites (Figure 1.6b), with the calixarenes acting as mediators of the PPIs required for crystallisation.\textsuperscript{26}

The Neri group also generated calix[4]arene derivatives, functionalised with tetrameric peptides, to bind to and inhibit the acyl transfer enzyme transglutamase (with up to 62 % reduction in activity), by blocking the entrance of the substrate to the active site.\textsuperscript{27} The Hof group used calixarenes along with readily-available dyes to form dye-displacement sensors for anti-body free reading of histones through lysine side chain recognition.\textsuperscript{28}

1.2.1.2 Resorcinarenes

Uchiyama and coworkers developed the use of resorcinarene scaffolds for histone surface recognition (Figure 1.7).\textsuperscript{29–32} Histones are basic proteins, containing many lysine residues, so the development of the resorcinarene compounds with many carboxylic acids aids their binding. They first developed compounds with 8 (monomeric) and 28 (tetrameric) surrounding carboxylates (4),\textsuperscript{29} followed by a more extended scaffold with 84
carboxylates,\textsuperscript{30} which were shown to bind to mixed histone proteins with $K_a$ $4.2 \times 10^5$ M$^{-1}$, $1.3 \times 10^7$ M$^{-1}$, and $8.4 \times 10^7$ M$^{-1}$ respectively by a surface plasmon resonance (SPR) assay. They were also shown to be selective for histones over lysozyme and ovalbumin, with binding increasing with increasing numbers of carboxylates.\textsuperscript{30} They later, transformed their tetrameric (28 carboxylate) ligand (4) into one that could be used in fluorescence intensity assays, by changing one of the resorcinarene units to a fluorescent dansyl group. The fluorescence of this dansyl group showed a 5-fold increase in fluorescence intensity on saturation with histone, and indicated the dansyl moiety as being located on a non-polar region of the histone surface.\textsuperscript{31} The binding affinity was found to be in the region of $10^6$ M$^{-1}$ with 1:1 binding demonstrated by this fluorescence intensity assay, and SPR. The $K_a$ was found to be higher at lower ionic strengths, thus implicating electrostatics as a major driving force for binding. The binding to $\sim 70$\% acetylated histone was also shown to be negligible.\textsuperscript{31} Further modifications to the tetrameric ligand (4) yielded a rotaxane wheel for a 2,6-disubstituted naphthalene thread with two fluorophore (fluorescein and rhodamine) moieties on either end.\textsuperscript{32} These showed similar fluorescence intensity profiles to the dansyl substituted compounds with $K_a$ $2.3 \times 10^6$ M$^{-1}$ and $9.0 \times 10^6$ M$^{-1}$ respectively, and large polarisation values.\textsuperscript{32} The compounds were similarly found to be selective over a variety of other proteins and were used in initial studies for FRET detection of histones.\textsuperscript{32}

\textbf{Figure 1.7} Uchiyama’s tetrameric resorcinarene compound (4) for binding to histone surfaces
The Botta group have also used dipeptide substituted resor[4]arenes for binding to human serum albumin (HSA) and α-ChT.\textsuperscript{33} With HSA there was a change in the circular dichroism (CD) spectrum for the resorcinarene but not for the protein showing there is an interaction but it does not affect the structural integrity of HSA. An enzyme assay showed they acted as non-competitive inhibitors of α-ChT with a non-denaturing gel showing the formation of a new species between α-ChT and the resorcinarene compounds.

1.2.1.3 Porphyrins

Since 1950, studies have investigated porphyrins binding to proteins; initially this was focussed on binding to HSA,\textsuperscript{34–36} but subsequently, porphyrins have been used for binding to a variety of different proteins, including K\textsubscript{v} potassium channels,\textsuperscript{37–41} VEGF,\textsuperscript{42} cyt c\textsuperscript{43,44} and lectins,\textsuperscript{45–47}

Trauner and coworkers rationally designed a tetraphenylporphyrin-based scaffold (5) to target the K\textsubscript{v} potassium channel with nanomolar affinity (Figure 1.8a), reducing the current through the channel.\textsuperscript{38} The C\textsubscript{4} symmetry of the porphyrin 5 was thought to be well-suited to the tetrameric nature of the potassium channel;\textsuperscript{38} however, solid state NMR has since shown that porphyrin 5 does not interact with all four subunits of the eukaryotic K\textsubscript{v}1.3 channel simultaneously,\textsuperscript{37,48} and instead lies perpendicular to the protein, projecting one of its cationic side chains into the channel rather than interacting with the surface of the channel.\textsuperscript{37} Porphyrin 5 has since been shown to block the ion conduction pathway and stabilise a closed K\textsubscript{v} channel state upon interaction with the voltage sensor domain.\textsuperscript{41} The Nolan group expanded on this work, looking at inhibiting specific K\textsubscript{v}1 channels, using a structure activity relationship (SAR) study to find ligands selective for the K\textsubscript{v}1.1 and K\textsubscript{v}1.2 channels.\textsuperscript{49}

![Figure 1.8 Porphyrin protein surface mimetics](image)

Figure 1.8 Porphyrin protein surface mimetics a) Porphyrin ligand of the K\textsubscript{v} potassium channel, b) Porphyrin ligand of FGF
The Yayon group have studied other porphyrins (6) that bind to fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (Figure 1.8b), with low micromolar affinity \textit{in vitro}, \textit{in cellulo}, and \textit{in vivo}. They also showed they were selective inhibitors of the VEGF/VEGF receptor PPI over the EGF/EGF receptor PPI. Further analysis led to the elucidation of higher affinity ligands with cationic porphyrins having the highest affinity \textit{in cellulo}.\textsuperscript{42}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure19.png}
\caption{Protein crystal structure of tetrasulfonatophenyl porphyrin bound to Jacalin (PDB ID: 1PXD)}
\end{figure}

The binding of porphyrins to lectins has been extensively studied with crystal structures having been solved for tetrasulfonatophenyl porphyrin binding to Jacalin (Figure 1.9),\textsuperscript{50} peanut lectin (PNA),\textsuperscript{51} and concanavalin A (ConA),\textsuperscript{52} showing different binding modes in each, highlighting the utility of porphyrins in protein binding.

\subsection{Dendrimers}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure110.png}
\caption{Schematic of a dendrimer, showing the core, branches and terminal (binding) groups}
\end{figure}
Dendrimers are supramolecular scaffolds of high valency, with a central core that projects a branching network of repeating units culminating in terminal functionality which can be used for binding to proteins (Figure 1.10).53 Twyman and coworkers have designed poly anionic poly(amidoamine) (PAMAM) dendrimers (Figure 1.11, 8) which bind to cyt c and α-ChT.54 The best ligands for both proteins, are those whose maximum addressable surface area matches the interfacial surface area of the protein. They have also shown that these dendrimers do not effect conformational changes in the structure of either protein.55

PAMAM dendrimers have also been shown to bind to HSA in an extensive study by the Giri group.56 They studied binding constants, NMR (1H, STD and DOSY) and molecular dynamic (MD) simulations of 19 PAMAM dendrimers in order to gain insight into the interactions, looking at differences in core, dendrimer generation and terminal group. This allowed for an analysis of the effect of hydrogen bonding, hydrophobicity, and electrostatic interactions on the binding to HSA. The NMR and MD simulations show that the inner shell protons of the dendrimer interact more strongly with the protein, compared to the outer protons.

![1st generation PAMAM dendrimer]

**Figure 1.11** 1st generation PAMAM dendrimer

**1.2.1.5 Other scaffolds**

The Hamilton group have also investigated anthracene receptors which bind to cyt c and lysozyme (Figure 1.12).57 The anthracene analogues (9) have a hydrophobic core surrounded by carboxylic acids to enable complementarity with the cyt c and lysozyme...
surfaces. 0.66 and 0.52 μM binding affinities (5 mM sodium phosphate, pH 7.4 buffer) were observed for cyt c and lysozyme respectively using a fluorescence quenching assay for cyt c and a Förster resonance energy transfer (FRET) assay for lysozyme. Thus implicating a different, but not further developed, surface mimetic scaffold.

![Figure 1.12 Anthracene protein surface mimetic scaffold](image)

Bivalent cyclodextrins have been synthesised, by Breslow and coworkers, to inhibit aggregation of citrate synthase and L-lactate dehydrogenase, by binding to (and thus preventing the aggregation of) hydrophobic patches on the protein surfaces. This was demonstrated by enzyme activity assays, non-denaturing size exclusion chromatography and chemical crosslinking.58

Kano and Ishida also developed a polyanionic β-cyclodextrin capable of binding to cyt c.59 Initial binding studies by isothermal titration calorimetry (ITC) show 1:1 entropically driven, electrostatic binding, with NMR suggesting binding at the haem exposed edge. Further confirmation and proposals for other binding sites were obtained by observation of partial inhibition of ascorbate reduction, and a decrease in reduction rate of cyt c in the presence of cyt c reductase. Additional studies involved making a more complicated ternary complex with a porphyrin spanning two cyt c bound cyclodextrins.

1.2.2 Metallo protein surface mimetics

1.2.2.1 Advantages of using metal complexes

Metals can offer advantages over conventional organic scaffolds, including the ability to offer a wider variety of coordination numbers and geometries, thus expanding the number of globular shapes available, allowing the potential for them to fit into pockets and onto surfaces not accessible to small organic molecules.60 Metal complexes can also exist as many more stereoisomers than organic molecules, for example an sp³ carbon with 4 different substituents has only two possible stereoisomers whereas an octahedral metal centre with 6 different ligands can exist in up to 30 different stereoisomers (Figure 1.13).61 The protein binding selectivity of small molecules has been shown to correlate to both their shape and stereochemical complexity,62 thus highlighting how the complexity of
a metal coordination complex can potentially be used for the generation of selective protein ligands.

Figure 1.13 Difference between sp³ carbon and octahedral metal scaffolds a) An sp³ carbon with 4 substituents can exist as two possible enantiomers, b) Examples of the 30 possible stereoisomers for an octahedral centre with 6 different substituents, c) fac, mer, Δ and Λ isomers on an octahedral centre can also exist, particularly when using unsymmetrical bidentate ligands

The use of metal complexes allows for combinatorial synthesis in order to generate a wide range of metal complexes using similar reactions, allowing the screening of a variety of compounds more easily. The metal centre itself can be used solely as a scaffold, for forming coordinative bonds with biological macromolecules, and for its reactive capacity, thus expanding the scope of possible binding interactions. Ligands can play a large role in redox behaviour, biostability, absorption and delivery of the metal complex. The ligands can also be used to direct the synthesis towards particular stereoisomers, for example by utilising the trans effect.

The use of metal scaffolds as molecular sensors also offers advantages over conventional small molecules, as it is possible to choose a metal scaffold which can itself be visualised using its intrinsic luminescence, rather than requiring functionalization of the organic framework, which may have an effect on binding, and other molecular properties, such as solubility. For example ruthenium(II) and iridium(III) complexes are phosphorescent, allowing for their direct visualisation in both biological assays and for cellular imaging.

1.2.2.2 Metal coordination to protein surfaces

Metal-ligand interactions in water are stronger than the conventionally important protein recognition interactions such as hydrogen-bonding, electrostatics and van der Waals contacts. This makes metal-ligand interactions a potentially useful tool in the
recognition of proteins, as fewer interactions will be needed to achieve high affinity, selective binding. The scope of this approach, however, is limited to certain amino acids and post-translational modifications, which are able to coordinate to a metal centre.

![Figure 1.14](image)

**Figure 1.14** Cu(II)-IDA for binding to protein surfaces, a) Structure of Cu(II)-iminodiacetate for binding to surface exposed histidines on bovine erythrocyte carbonic anhydrase, b) Protein crystal structure of a Cu(II)-IDA bound to a histidine on human carbonic anhydrase II (PDB ID: 2FOV)

Mallik and coworkers used the knowledge that many transition metals bind to the imidazole side chains of histidines on proteins, a widely used concept in the purification of proteins by immobilized metal affinity chromatography. They used molecules with copper(II)-iminodiacetate (IDA) arms to recognise patterns of surface-exposed histidine residues (Figure 1.14), resulting in recognition of bovine erythrocyte carbonic anhydrase, after seeing that they could be used to bind to histidines on peptides in solution. A three Cu(II) system (10) was used to bind three histidine side chains on the N-terminus of the carbonic anhydrase, with the ligand alone showing no binding, illustrating the importance of the metal centre for recognition. The highest affinity compound (10, 3 μM K_d by ITC) was also found to be selective for carbonic anhydrase over chicken egg albumin, a protein with the same number of surface exposed histidine residues (six) but in different spatial orientations.

Along similar lines, Hamachi and coworkers used bis(Zn(II)-dipocolylamine (Dpa))) derivatives to bind histidine residues on the surface of α-helical peptides, stabilising their α-helical conformations. This lead to the use of the bis(Zn(II)-Dpa) complexes in the binding of both mono- and multi-phosphorylated peptides via bidentate binding between the Zn(II) and the phosphate groups, resulting in stabilisation of the peptides (Figure 1.15). The targeting of phosphate groups on protein surfaces is of particular interest.
as protein phosphorylation is used as a ubiquitous signaling mechanism within cells so binding preferentially to a phosphorylated state of a protein may allow modulation of signaling pathways.

**Figure 1.15** bis(Zn(II)-(dpa)) species bound to two phospho-amino acid residues, stabilising the α-helical conformation

The bis(Zn(II)-Dpa) species have been used to generate chemosensors tailoring the bridging group between the two Zn(II) centres to cause a change in fluorescence on binding, in Hamachi’s case this involved using anthracene or bipyridine moieties, thus allowing for fluorescence binding assays to be developed.\(^{74-77}\) Using di-phosphorylated model α-helical peptides it was shown, by CD, that appropriately spaced Zn(II) centres increased the α-helical content of the peptide. They showed 10-fold selectivity for di-phosphorylated over mono-phosphorylated peptides, but little selectivity between different di-phosphorylated peptides, due to the high flexibility in the linker between the two metal binding sites.\(^{75,78}\) This has subsequently been used to inhibit the phospho-PPI between the phosphorylated CTD peptide and the Pin1 WW domain with 5.6 μM affinity.\(^{79}\)

A more rigid diazastilbene linker has since been used to generate a receptor for \((i, i+1)\) di-phosphorylated peptides, which gives different luminescent responses for different spatial relationships between the phospho-amino acids residues.\(^{80}\) Zn(II) complexes based on these scaffolds have also been coupled to a \(\text{bis}[(4,6\text{-difluorophenyl})\text{pyridanto-}N,C^2]\) iridium(III) picolinate to generate a phosphorescent sensor for phosphorylated peptides, with much better selectivity over ATP.\(^{81}\)

Following on from Hamachi’s work, Gunning and coworkers used Cu(II)-\(\text{bis}(\text{Dpa})\) and Zn(II)-\(\text{(Dpa)}\) complexes to bind to phospho-tyrosine STAT3, inhibiting STAT3/STAT3 dimerisation.\(^{82,83}\) ITC and fluorescence polarisation data showed the Cu(II) complexes binding to a phospho-peptide (with micromolar \(K_d\)), inhibiting the phospho-peptide-protein complex, with micromolar \(K_i\).\(^{82}\) The Cu(II) complexes were further shown to inhibit STAT3/STAT3-DNA binding, using an electrophoretic mobility shift assay, with 8.2 μM affinity for the highest affinity ligand. They also exhibited low micromolar IC\(_{50}\) in 3 different cancer cell lines but much lower inhibition in, and low cytotoxicity in healthy
NIH3T3 cells, thus showing their potential therapeutic utility. Later, they showed the use of bis(Zn(II)-(Dpa)) complexes as similar mimics of src homology domain 2 (SH2) domains showing, by fluorescence quenching experiments, that the Zn(II) complexes could bind phospho-tyrosine peptides, with $K_d \sim 10^{-7}$M and some sequence identity discrimination. Some of the Zn(II) compounds were shown to be cytotoxic in three types of cancer cell, but with some inconsistencies with the fluorescence binding data.

### 1.2.2.3 Metalloporphyrins

Considerable research was performed in the 80s and 90s on electron transfer between both metallo and non-metallo anionic porphyrins and cyt c. With two types of porphyrins being compared: uroporphyrins and 4-carboxyporphyrins by Jameson et al. in 1997. 4-Carboxy porphyrins were shown to have higher fluorescence quenching rates, probably due to the difference in orientation for the two porphyrins, which can be visualised by the induced CD of the porphyrins on binding to cyt c. Interestingly these original porphyrins do not affect the CD of cyt c at room temperature, indicating that there is no change in protein tertiary structure on binding. The Rodgers group also used cationic metalloporphyrins as extrinsic probes to study peptide aggregation by analysing photoinduced electron transfer (PET) from tyrosine or tryptophan residues in the protein to the metalloporphyrin.

Following Fisher’s initial observation that tetra-carboxy phenyl porphyrin bound to cyt c, selectively over acetylated cyt c, with $K_d$ in the region 0.05 μM – 5 μM using a flavodoxin competition assay, the Hamilton group developed higher affinity cyt c tetra-phenyl porphyrin ligands. Tetra-phenyl porphyrin scaffolds provide a large, flat, semi-rigid molecular surface of ~300 – 400 Å$^2$ which with anionic substituents on the periphery bind to cyt c in a 1:1 stoichiometry. They developed first nano- then subnano-molar receptors for cyt c and showed that increasing the number of carboxylates on the periphery led to significant increases in binding affinity. They also showed that there were changes in affinity by altering the relative proportions of acidic and aromatic functionalities. The compounds were found to be selective for cyt c over the related proteins, cyt c$_{551}$ and ferredoxin. Crowley and coworkers later analysed sulfonatoporphyrins binding to cyt c by $^1$H-$^{15}$N HSQC NMR, with the results corroborated by molecular docking. They showed that a dynamic ensemble of energetically similar interactions exists, with the porphyrin being able to move over different patches on the cyt c surface. The Hamilton group have also used functionalised porphyrins to design an array for protein detection.
Metalloporphyrins for binding to cyt c a) The two best cyt c binders and denaturants, b) schematic of the Cu(II) porphyrin dimers

Interestingly the compounds were found to denature cyt c, shown by a lowered melting temperature \( (T_m) \),\(^{96} \) by up to 50 °C.\(^{44} \) The anionic tetra-phenyl porphyrins (11 and 12, \( M = 2H \), Figure 1.16a) had a more profound effect compared to similarly charged molecules and porphyrins without the phenyl group; this could perhaps be seen as unsurprising as studies with different anionic polymers on their denaturing effect on cyt c showed that a hydrophobic portion is required for observable changes in cyt c stability.\(^{97} \) The porphyrins do not show a denaturing profile for acetylated cyt c or cyt c\(_{551} \), showing that charge complementarity is key, though higher affinity binding to cyt c does not necessarily make for a better denaturant. This is likely because the porphyrin is thought to destabilize the native state and stabilize the unfolded state of the protein, so some of the higher affinity ligands may bind better to the native state than the lower affinity binders, stabilising the native state comparatively. The compounds also show increased trypsin digest rates of cyt c, even with only 0.1 equivalents of porphyrin to protein.

After studying free base porphyrins, the Hamilton group analysed metalloporphyrins, in particular Cu(II) porphyrins.\(^{98,99} \) These are known to dimerise in aqueous solutions due to enhanced \( \pi-\pi \) stacking (Figure 1.16b).\(^{100,101} \) The Cu(II) porphyrins, were shown to have 60 nM affinity for cyt c and bind in a 2:1 porphyrin:protein ratio, with the porphyrins maintaining their dimeric nature. The Cu(II) porphyrins (unlike the monomeric Zn(II) equivalents) were shown to denature cyt c, first stoichiometrically\(^{98} \) and then catalytically (0.1 eq.),\(^{99} \) with some able to do so at room temperature. This denaturation was selective for cyt c over \( \alpha \)-lactalbumin, Bcl-x\(_L \), cyt c\(_{551} \), myoglobin and RNase A. The denaturation was shown to unravel the \( \alpha \)-helical nature of the protein as well as increase the tryptic digest.
rates. There are significant differences between the free base, where there is some lowering of the $T_m$, and the Cu(II) porphyrins, which have much larger effects arising from the dimeric nature of the Cu(II) porphyrins compared to the monomeric free base porphyrins ($M = 2H$). In order to bind to the native cyt $c$ structure the Cu(II) porphyrin dimers may have to dissociate, whereas to bind to the denatured cyt $c$ they do not, this creates a catalytic cycle whereby the Cu(II) porphyrin catalyses the denaturation. After studying cyt $c$ denaturation they decided to assess other haem proteins and showed that Cu(II) porphyrins can also be used to denature both myoglobin and haemoglobin.$^{102}$

Zn(II) and Fe(III) metalloporphyrins have also been shown to multimerise cyt $c$ from *Geobacter sulfurreducens*, hen egg lysozyme and horse heart cyt $c$ at high (millimolar) porphyrin and protein concentrations,$^{103}$ as observed by SAXS and corroborated by MD calculations.

### 1.2.2.4 Metalloporphyrins

Zn(II) porphyrin-based dendrimers have also been developed, with the fluorescent metalloporphyrin-core being utilised for detection ([Figure 1.17].$^{104}$ These large multivalent nanoscale structures have been used to bind to cyt $c$, with the cyt $c$/dendrimer complex being more stable than the native cyt $c$/cyt $b_5$ PPI, evidenced by 20 % fluorescence recovery on addition of 14 equivalents of cyt $b_5$ to the cyt $c$/dendrimer
complex. One of these original Zn(II)-porphyrin dendrimers (13), and subsequent generations, were shown to improve cell viability when cells are subjected to an apoptotic stimulus.\textsuperscript{105} It has been hypothesised that the dendrimers trap cyt c, preventing it from interacting with Apaf1 to form the apoptosome, thus inhibiting apoptosis.

1.2.2.5 Transition metal complexes

1.2.2.5.1 Meggers Ruthenium(II) complexes

![Figure 1.18 Meggers Ru(II) based protein kinase ligands](image)

Figure 1.18 Meggers Ru(II) based protein kinase ligands a) Structures of staurosporine (14), a pan kinase inhibitor and Ru(II) complexes based on it for binding to GSK-3β (15), MSK-1 (16) and PAK-1 (17), b) Protein crystal structure of a Ru(II) complex bound to PAK-1 (PDB ID: 3FXZ)

The use of metals as scaffolds for protein binding molecules has been pursued by many groups, with the Meggers group being a front-runner. They have mainly used ruthenium(II) complexes (Figure 1.18), but more recently have branched out to using rhodium(III),\textsuperscript{106,107} iridium(III),\textsuperscript{108,109} osmium(II)\textsuperscript{110} and platinum(II)\textsuperscript{111} for the inhibition of many different protein kinases including Pim1,\textsuperscript{112,113} glycogen synthetases kinase 3β (GSK3β),\textsuperscript{114} MSK1,\textsuperscript{112} BRAF kinase,\textsuperscript{115} and PAK1.\textsuperscript{116} X-ray crystal structures have been solved for many of these compounds bound to their target kinases (Figure 1.18b), showing the metals acting in purely structural capacities.\textsuperscript{113,117} The majority of these have been adenosine triphosphate (ATP) mimics, being based on staurosporine (14), a widely studied organic ATP mimic, but non-ATP mimics have been studied more recently,\textsuperscript{118} as have inhibitors of other nucleotide binding proteins including the human repair enzyme 7,8-dihydro-8-oxoguanosine triphosphatase,\textsuperscript{119} and the lipid kinase PI3K.\textsuperscript{120}
However these studies have all looked at ‘small’ metal complexes fitting into defined pockets (ATP binding sites) on the protein rather than the protein surface; in order to bind to protein surfaces it is more appropriate to consider larger supramolecular scaffolds.

### 1.2.2.5.2 Inert Group 9 complexes

![Figure 1.19](image_url) 

**Figure 1.19** Inert group 9 protein surface ligands a) TNF-α ligand, b) Improved TNF-α ligand, c) STAT3 ligand d) hDM2 ligand

The Leung group have worked on multiple Ir(III) and Rh(III) compounds, capable of binding to protein surfaces. They first developed cyclometalated Ir(III) complexes capable of binding to tumour necrosis factor-α (TNF-α). The Ir(III) complex developed utilises the aromatic bidentate ligands 2-phenylpyridinato (ppy) and 2,2'-biquinoline (biq) (**Figure 1.19a**), in order to target a hydrophobic binding site on the TNF-α dimer, preventing active trimer formation. Both enantiomers of the Ir(III) complex were found, by ELISA, to have an IC₅₀ in the region of 20 μM, comparable to that of SPD304, one of the strongest inhibitors of TNF-α. Structure-activity relationships have since been performed, using 22 Ir(III) complexes with ligands of different shapes and sizes in order to generate low micromolar inhibitors (**Figure 1.19b**)(seen in an *in cellulo* inhibition of TNF-α induced NF-κB luciferase assay in HEP G2 cells). They also looked at the effect of stereochemistry, comparing the Δ and Λ isomers, showing that the Λ isomers had increased cellular activity (3.4 μM versus 9.9 μM IC₅₀ in the cellular assay) and binding affinity (30 versus 57 μM IC₅₀ in an *in vitro* assay).

They have synthesised Ir(III) and Rh(III) compounds capable of binding to, and preventing dimerization and phosphorylation of STAT3 (**Figure 1.19c**). The most
potent Rh(III) compound (20) was found to have anti-tumour activity in a mouse xenograft tumour model and was found to bind to the SH2 domain of STAT3 with an IC₅₀ of 4.8 μM. STAT3 pull-down assays showed an inhibition of STAT3 dimerisation and Western blotting showed an inhibition of STAT3 phosphorylation. The group have also screened a series of iridium(III) complexes as inhibitors of the p53/MDM2 interaction (Figure 1.19d). One compound (21) was shown to be a 16 μM inhibitor in a fluorescence anisotropy competition assay. Subsequent cellular analysis confirmed the induction of p21 (a downstream target of p53) and apoptosis.

Figure 1.20 Irreversible group 9 metal complex protein surface binders a) BRD4, b) Aβ₁₋₄₀

More recently they have developed an Ir(III) based irreversible inhibitor (22, Figure 1.20a) of the interaction between bromodomain-containing protein 4 (BRD4) and an acetylated histone peptide. They initially screened 27 compounds and found a compound capable of modulating the interaction between BRD4 and chromatin in vitro and in vivo. The compound was found to bind to histidine residues, with the loss of acetonitrile ligands, and was found to be selective over the other histidine containing proteins STAT3 and caspase-6. They have also developed Ir(III) and Rh(III) complexes (Figure 1.20b) that inhibit the aggregation of Aβ₁₋₄₀, a peptide implicated in neurodegeneration in Alzheimer’s disease. The compounds bind to histidine residues in the peptide, replacing the water ligands with these histidines, allowing further interactions of the hydrophobic coligands with hydrophobic residues at the N-terminus of the peptide. The compounds can be used as luminescent probes for the Aβ₁₋₄₀ peptide and so offer an approach to the study of this important peptide.

1.2.2.5.3 Metal tris (bipyridines)

In the 1950s and 60s Dwyer and coworkers showed that simple bipyridine (bpy) and phenanthroline (phen) ruthenium(II) complexes show bacteriostatic and bacteriocidal activities and also inhibit tumour growth, thus showing the potential biological utility of these kinds of complex. Sasaki et al. further reported, in 1993, a saccharide
substituted Fe(II)(bpy)$_3$ complex capable of binding to lectins,$^{130}$ thus opening the idea of using metal *tris* (bipyridines) for binding to proteins.$^{130}$

![Schematic of DCC based on Fe(II)(bpy)$_3$ complexes](image)

**Figure 1.21** Schematic of DCC based on Fe(II)(bpy)$_3$ complexes

Fe(II)(bpy)$_3$ complexes are relatively dynamic in aqueous solution, this allows for the use of dynamic combinatorial chemistry (DCC) around the Fe(II) core (**Figure 1.21**). This has been used by the Sasaki and de Mendoza groups in order to generate lectin binding complexes.$^{131,132}$ Sasaki and coworkers generated an Fe(II)(bpy)$_3$ complex with mono-GalNAc substituted bpy ligands, which altered its stereochemical configuration in solution resulting in the enrichment of higher affinity compounds for various different lectins.$^{131}$ De Mendoza and co-workers used bipyridines functionalised with 3 different sugars, complexed them to Fe(II) then incubated the Fe(II)(bpy)$_3$ complexes with the mannose-binding lectin, Con A, this, as predicted, resulted in the enrichment of the mannose functionalised complex (detected by LCMS)$^{132}$.

While the labile nature of the Fe(II)(bpy)$_3$ complexes can be useful for the generation and selection of high affinity protein binders, the inert nature of Ru(II)(bpy)$_3$ is of great interest, as decomplexation will not occur in biological media in dilute solution.$^{133,130}$ Kaboyashi and coworkers,$^{133,134}$ generated a series of glyco-functionalised Fe(bpy)$_3$ and Ru(II)(bpy)$_3$ compounds, and showed that the ruthenium(II) glyoclusters had high lectin affinity and increased luminescence on lectin binding. The Seeberger group have since developed sugar functionalised Ru(II)(bpy)$_3$ complexes ($24$) that bind to the concavalin A (ConA) and *galanthus nivalis agglutinin* (GNA) (**Figure 1.22**), by an electron transfer assay.$^{135}$ This was followed by a study using digital logic analysis to determine the best
lectin binders for further study, by assessing the increase in luminescence output of the Ru(II)glycodendrimers with various different lectins. The Ru(II)(bpy)$_3$ complexes with surface bound lectins have also been used as luminescent sensors for measuring monosaccharide and oligosaccharide concentrations, by using the displacement of the Ru(II)glycodendrimers from the lectin surface by the sugars. They then further functionalised their Ru(II)(bpy)$_3$ complex scaffold, by addition of adamantane units, and adding mannose functionalised β-cyclodextrin to encapsulate the adamantane units, thus making a highly mannose functionalised subunit which binds to high density ConA with 0.14 μM $K_d$ as determined by SPR. The Okada group have also looked at sugar functionalised Ru(II)(bpy)$_3$ complexes and have shown that galactose functionalised

Figure 1.22 Seeberger’s mannose functionalised Ru(II)(bpy)$_3$ complex for ConA/GNA binding
Ru(II)(bpy)₃ complexes bind to peanut agglutinin with 6.1 μM Kₐ and glucose functionalised Ru(II)(bpy)₃ complexes bind to ConA with 18 μM Kₐ by both fluorescence emission and polarisation assays.¹³⁹

Electron transfer experiments between cyt c and Ru(II)(bpy)₃ complexes (as well as Ru(II)(phen)₃, Os(II)(bpy)₃ and Os(II)(phen)₃ complexes) were initially reported by Cho in the 1980s.¹⁴⁰ Subsequently Hamachi and coworkers reported carboxylate functionalised Ru(II)(bpy)₃ derivatives (25, Figure 1.23) that could bind to, and photoreduce cyt c (pI = 10.0) selectively over other proteins (myoglobin, horseradish peroxidase and cyt b₅₆₂) with lower pIs (7.0, 8.0 and 5.0 respectively).¹⁴¹ The Ru(II)(bpy)₃ complexes 25 were found to bind to cyt c, by an ultrafiltration binding assay. The compound with the highest number of carboxylic acids (18) was shown to bind an order of magnitude better than an unfunctionalised Ru(II)(bpy)₃ complex. The Ru(II)(bpy)₃ complexes were capable of photoreducing cyt c with the most effective being an asymmetric Ru(II)(bpy)₃ complex with 12 carboxylates rather than the fully functionalised Ru(II)(bpy)₃ complex with 18 carboxylates.¹⁴¹

![Figure 1.23](image)

Figure 1.23 Hamachi’s carboxylate functionalised Ru(II)(bpy)₃ complexes for cyt c binding and photoreduction

Following on from Hamachi’s initial observations both the Ohkanda and Wilson groups further established selective binding of Ru(II)(bpy)₃ complexes to cyt c and α-ChT (Figure 1.24). The Wilson group developed both mono- (5’) (27a) and di- (4,4’) (26a) substituted bpy moieties which, when complexed to ruthenium(II), show 1.6 nM binding affinity (26a, 5 mM sodium phosphate, pH 7.4) for cyt c by a luminescence quenching assay.¹⁴²,¹⁴³ As with Hamilton’s porphyrins,⁴³ negatively charged substituents (based on aspartic acid moieties) show good binding in luminescence quenching assays.¹⁴² Negative cooperativity was observed, with increasing numbers of carboxylates, the binding affinity per carboxylate decreases. Geometrically the mer isomers of the 5’ mono-substituted Ru(II)(bpy)₃ complexes (27a) showed ~10 fold better binding affinity (25 vs. 172 nM for
the Δ isomers, 5 mM sodium phosphate, pH 7.4) than the fac isomers for cyt c, but the Δ and Λ isomers showed little difference in their binding affinities (25 vs 29 nM for the mer isomers). Further analysis by a functional ascorbate assay showed that both the (4,4') disubstituted and 5' monosubstituted bipyridines slow the rate of reduction of cyt c, probably as a result of blocking the approach of the reducing agent to the haem group, which is surrounded by basic amino acid residues. The absence of binding to 60 % acetylated cyt c confirms that the charge complementarity is key to binding. The binding of the Ru(II)(bpy)₃ complex 26a, similarly to Hamilton’s porphyrins, lowers the melting temperature of cyt c by 25 °C and shows an increased rate of proteolytic degradation in both stoichiometric and substoichiometric quantities of the Ru(II)(bpy)₃ complex. At higher temperatures there is a change in binding stoichiometry observed with a change from 1:1 binding to 2:1 (protein:Ru(II)(bpy)₃ complex) binding being observed on changing from 25 to 70 °C. In cellulo work has also been performed with the 4,4'-disubstituted Ru(II)(bpy)₃ complexes showing 95 % efficiency of transfection into HEK-293T cells at 10 μM concentration. The Ru(II)(bpy)₃ complexes appear to localise in the lysosomes and in several instances are non-cytotoxic.

![Figure 1.24](image_url) Ru(II)(bpy)₃ complexes designed by the Wilson and Ohkanda groups for binding to cyt c (26a, 27a) and α-ChT (26b)

Ohkanda and co-workers have shown that similar dendritic Ru(II)(bpy)₃ complexes (26b, Figure 1.24) bind to α-ChT in a mixed 1:1 and 1:2 (Ru(II)(bpy)₃ complex:α-ChT) stoichiometry with 130 and 430 nM Kᵦₕ (5 mM phosphate, pH 7.4) for the first and second equilibrium step respectively, inhibiting the enzyme by non-competitive inhibition. They later synthesised heteroleptic Ru(II)(bpy)₃ complexes, containing multiple bipyridine ligands, for binding to both α-ChT and cyt c, with submicromolar affinity. Molecular dynamics showed that 3 isophthalic arms interact with α-ChT, and 4 isophthalic
arms interact with cyt c.\textsuperscript{147} In cellulo work also showed the Ru(II)(bpy)\textsubscript{3} complexes being able to enter cells.\textsuperscript{147}

1.3 Project aims

As described previously Ru(II)(bpy)\textsubscript{3} complexes have been rationally designed for binding to protein surfaces, including to lectins, cyt c and α-ChT, however little is known of how these Ru(II)(bpy)\textsubscript{3} complexes interact with proteins; the scope of these Ru(II)(bpy)\textsubscript{3} complexes for binding to protein surfaces and if we can use methods other than rational design to obtain novel and unsymmetrical Ru(II)(bpy)\textsubscript{3} complexes for binding to proteins.

The aims of this project were to further develop the Ru(II)(bpy)\textsubscript{3} scaffold in order to discover its utility for protein surface recognition. This involved-

- Furthering the understanding of the binding of previously designed Ru(II)(bpy)\textsubscript{3} to the surface of cyt c, by elucidating more information about the binding mode and the interactions involved in binding, as well as the location of the binding sites. This allowed a more complete understanding of how Ru(II)(bpy)\textsubscript{3} complexes bind to proteins, to allow for further rational design for binding to other, more therapeutically interesting, protein surfaces.
- The development of a suitable scaffold for dynamic combinatorial chemistry in order to generate higher affinity, and less symmetrical Ru(II)(bpy)\textsubscript{3} complex, and porphyrin binders for different proteins.
- The design of a protein sensing array using Ru(II)(bpy)\textsubscript{3} complexes to discriminate between a variety of different protein surfaces, to give another potential protein surface binding application of these Ru(II)(bpy)\textsubscript{3} complexes.
2 Biophysical and Structural Studies on Cytochrome c Recognition with Functionalised Ruthenium(II) Tris (bipyridine) Complexes


Cytochrome (cyt) c is a highly abundant, well-studied protein, which can be bought in gram quantities; this makes it an ideal protein for the study of how different molecular scaffolds can be used to bind to protein surfaces. In this work, cyt c has been used as a model protein for understanding how Ru(II)(bpy)$_3$ complexes bind to the surface of proteins. The cyt c binding of a range of Ru(II)(bpy)$_3$ complexes has been studied, followed by a more in depth study of the binding of two of these Ru(II)(bpy)$_3$ complexes to cyt c aimed at understanding how these Ru(II)(bpy)$_3$ complexes interact with cyt c, if they mimic the cyt c/cyt c peroxidase (CCP) PPI, and also to elucidate the binding site of the Ru(II)(bpy)$_3$ complexes on cyt c. Increased knowledge of how Ru(II)(bpy)$_3$ complexes bind to protein surfaces should allow for the design and use of novel Ru(II)(bpy)$_3$ complexes for binding to other, more therapeutically interesting, protein surfaces.

2.1 Cyt c and the cyt c/CCP PPI

Figure 2.1 The cyt c/CCP PPI structure (PDB ID 1U75).\textsuperscript{148} a) the cyt c/CCP PPI, b) the binding interface between cyt c and CCP showing the ring of basic amino acids (blue) on cyt c (red ring) and acidic amino acids (red) on CCP (blue ring) surrounding a central hydrophobic core on both proteins

Cyt c is a small, basic haem protein ordinarily located in the inner mitochondrial membrane. It is used within the electron transport chain of respiration, shuttling
electrons between cyt c reductase and cyt c oxidase. Cyt c is also implicated in apoptosis where, upon appropriate cellular signalling, it is released from the mitochondrion,149 binds to apoptosis protease activating factor-1 (Apaf-1), generating a signalling cascade which results in the cleavage of many key cellular proteins.150,151

Another of cyt c's native protein partners is cyt c peroxidase (CCP), which is found in the mitochondrial intermembrane space of plants and takes electrons from reduced cyt c to convert hydrogen peroxide into water. The cyt c/CCP protein-protein interaction (PPI) has been very well studied,152 it was the first non-antibody PPI to have its X-ray crystal structure solved (Figure 2.1a).153 The cyt c/CCP binding interface is located close to the haem exposed edges on both cyt c and CCP,153 this allows it to perform its function, transferring electrons between the two proteins. The binding is based on a central hydrophobic core of amino acids on both proteins, surrounded by basic amino acid residues on cyt c and acidic amino acid residues on CCP (Figure 2.1b).153 This allows for charge-charge complementarity between the two protein surfaces. The binding is very ionic strength dependent, indicating a large electrostatic driving force to binding, showing the importance of this charge-charge complementarity.154 The binding interaction is entropy-driven, and is even enthalpically unfavourable.155

2.2 Ru(II)(bpy)₃ complexes for binding to cyt c

Different multivalent scaffolds have been used to design molecules to bind to cyt c, many of these were discussed in Chapter 1. A defining feature of the design of these molecules is the addition of multiple carboxylic acid moieties to bind to the basic surface of cyt c.

![Figure 2.2 Ru(II)(bpy)₃ complexes designed for binding to cyt c](image-url)
It was decided to synthesise the Ru(II)(bpy)$_3$ complexes 26 and 29 - 35 (Figure 2.2) in order to test their binding to cyt c and to understand their protein binding interactions. Ru(II)(bpy)$_3$ complexes 26, 29, 31, 34 and 35 had previously been synthesised by the Wilson group for binding to cyt c. The Ru(II)(bpy)$_3$ complexes 26, 29 and 31 were functionalised with multiple carboxylic acid moieties in order to bind to the basic amino acid residues on cyt c. Ru(II)(bpy)$_3$ complex 34 possesses crown ether moieties was designed to potentially chelate the lysine residues on cyt c, however no binding was detected, and Ru(II)(bpy)$_3$ complex 35 was designed as a negative control, being amine functionalised it should not bind to the basic cyt c. In addition to the previously designed Ru(II)(bpy)$_3$ complexes, Ru(II)(bpy)$_3$ complexes 30 and 32 have been synthesised in order to further expand the number of Ru(II)(bpy)$_3$ complexes (Ru(II)(bpy)$_3$ complex 32 was synthesised by Georgina Pleasance) to see if the spatial location of the carboxylates affects binding affinity. The synthesis of Ru(II)(bpy)$_3$ complex 33 was also attempted, however the secondary amide bond was found to cleave during the ruthenium(II) complexation reaction. The initial thought behind the synthesis of Ru(II)(bpy)$_3$ complexes 32 and 33 was to systems with a similar cyt c binding profile to the previously designed Ru(II)(bpy)$_3$ complexes 26 and 31 that existed as enantiomers rather than diastereomers, potentially permitting attempts at protein crystal growing trials with the Ru(II)(bpy)$_3$ complexes.

2.2.1 Synthesis

The synthesis of these Ru(II)(bpy)$_3$ complexes was achieved as described previously (Scheme 2.1), with formation of the protected ligands 41 by amide bond formation on 4,4'-dicarboxylic acid-2,2'-bipyridine 40, via a particularly water sensitive diacid chloride. For the larger Ru(II)(bpy)$_3$ complexes (26, 33, 34 and 35), the functionalised anilines 38 for the amide bond formation were synthesised as in Scheme 2.1a, by amide bond formation, via the diacid chloride, on 5-nitro isophthalic acid 36, followed by hydrogenation of the nitro group to the aniline 38. These hydrogenation reactions could not be performed on large scale, as there was no reaction or incomplete reaction when performed on more than 1 g scale.

The bipyridine ligands 41 synthesised were then complexed to ruthenium(II) using Wilkinson’s reagent (Ru(II)(DMSO)$_4$Cl$_2$) to yield the protected Ru(II)(bpy)$_3$ complexes 42. These protected Ru(II)(bpy)$_3$ complexes 42 were purified by conventional silica or alumina column chromatography. This purification was changed from the previously reported ion exchange Sephadex columns, as Ru(II)(bpy)$_3$ complexes with large
hydrophobic protecting groups precipitated on these columns due to the aqueous NaCl eluents used.

Scheme 2.1 Synthesis of Ru(II)(bpy)₃ complexes 26, 29-35
a) Synthesis of isophthalimide anilines 38 for Ru(II)(bpy)₃ complexes 26, 31 - 35, b) Synthesis of final Ru(II)(bpy)₃ complexes, 
R(P) = protected R group

These protected Ru(II)(bpy)₃ complexes 42 were subsequently deprotected in an appropriate manner, dependent on their protecting groups (Table 2.1). It should be noted that the anilide bonds in Ru(II)(bpy)₃ complex 26 are activated towards hydrolysis in both acidic and basic conditions, meaning mild conditions were required for this deprotection, with both acid (tert-butyl ester) and base (methyl ester) labile protecting groups being
attempted. A methyl ester strategy with only 50 equivalents (~2 per acid) of lithium hydroxide and a short (1 hour) reaction time was eventually found to be sufficient for deprotection, without cleavage of the anilide bond.

<table>
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<th>Complex</th>
<th>Protecting group</th>
<th>Deprotection method</th>
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<td>Et</td>
<td>1 M NaOH + EtOH</td>
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<tr>
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<td>t-Bu</td>
<td>TFA/H$_2$O</td>
</tr>
<tr>
<td>32</td>
<td>Et</td>
<td>1 M NaOH + EtOH</td>
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<tr>
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<td>Boc</td>
<td>1 M HCl in dioxane</td>
</tr>
</tbody>
</table>

**2.3 Assay development**

Figure 2.3 Cartoon illustrating how the luminescence quenching assay works a) The Ru(II)(bpy)$_3$ complex on excitation with light at 467 nm consequently emits light at ~625 nm, b) When bound to cyt c, the emission is quenched by photoinduced electron transfer (PET) to the haem group of cyt c

The detection of binding between the Ru(II)(bpy)$_3$ complexes and cyt c was achieved by a, previously reported, luminescence quenching assay (Figure 2.3).$^{142,143}$ The Ru(II)(bpy)$_3$ complexes are luminescent, when excited by light at 467 nm, they consequently emit light at ~625 nm, however when bound to cyt c this luminescence is quenched by photoinduced electron transfer (PET) to the iron in the haem group of cyt c.
Titrating variable concentrations of cyt c into a fixed concentration of Ru(II)(bpy)$_3$ complex allows a binding isotherm to be established (Figure 2.4).

![Figure 2.4 Luminescence quenching assay](image)

**Figure 2.4** Luminescence quenching assay a) Graph showing the quenching of the luminescence of complex 26 upon addition of increasing concentrations of cyt c b) Fitting to 1:1 binding isotherm, in 5 mM sodium phosphate, pH 7.5

### 2.3.1 Assay development from fluorometer to plate reader.

Previously the luminescence quenching assays were performed on a fluorometer, however this does not allow for high-throughput screening, taking over 2 hours to measure the binding of one Ru(II)(bpy)$_3$ complex to cyt c in triplicate, therefore it was decided to develop the assay for use on a fluorescence plate reader. The use of a fluorometer allows for the use of quartz cuvettes, which have very little interaction with the components of the assay, as compared to the plastic plates used in conventional fluorescence plate reader assays, onto which binding of proteins and molecules is often observed.

Using the same conditions as for the fluorometer on a plate reader, did not produce an agreement in binding between the two machines, and data often did not fit a 1:1 binding isotherm (Figure 2.5a), indicating interactions between the plate and cyt c or the Ru(II)(bpy)$_3$ complex. This showed an additive to disrupt the interaction between the plate and the assay components was required.

Two commonly used buffer additives to disrupt these interactions are the detergent Tween20 and the blocking agent bovine serum albumin (BSA). Assays with these different additives were first attempted with a sample of Ru(II)(bpy)$_3$ complex 26, with a small amount of impurity from the cleavage of an anilide bond in deprotection. Upon attempting the assay with the Tween20 and BSA additives on both the plate reader and fluorometer (Table 2.2), there was still a large discrepancy between the fluorometer and plate reader.
when using Tween20, but there was little difference with the addition of BSA. Therefore BSA was added for all plate reader binding experiments. This decision was validated using a resynthesized clean sample of Ru(II)(bpy)$_3$ complex 26, where the $K_d$ values were 40.0 ± 4.5 nM and 17.5 ± 3.3 nM (50 nM Ru(II)(bpy)$_3$ complex 26, 5 mM sodium phosphate, 0.2 mg mL$^{-1}$ BSA, pH 7.5) for the plate reader and fluorometer respectively, whereas without BSA the binding curve on the plate reader did not fit to the 1:1 binding isotherm (Figure 2.5), but gave a $K_d$ of 10.5 ± 0.4 nM on the fluorometer, confirming the previously obtained data.

Table 2.2 Difference in cyt c binding affinity between the plate reader and fluorometer, all 100 nM Ru(II)(bpy)$_3$ complex 26, in 5 mM sodium phosphate, pH 7.4

<table>
<thead>
<tr>
<th>Buffer additive</th>
<th>$K_d$ fluorometer/ nM</th>
<th>$K_d$ plate reader/ nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No additive</td>
<td>7.39 ± 3.29</td>
<td>95.27 ± 10.78</td>
</tr>
<tr>
<td>0.05 % Tween 20</td>
<td>9.23 ± 1.79</td>
<td>113.22 ± 30.27</td>
</tr>
<tr>
<td>0.2 mg mL$^{-1}$ BSA</td>
<td>35.74 ± 7.19</td>
<td>49.62 ± 27.63</td>
</tr>
</tbody>
</table>

Figure 2.5 Binding of Ru(II)(bpy)$_3$ complex 26 to cyt c on plate reader in 5 mM sodium phosphate, pH 7.4 a) without BSA additive, b) with 0.2 mg mL$^{-1}$ BSA

2.4 Binding of different Ru(II)(bpy)$_3$ complexes

Having established an assay regime for more high-throughput binding cyt c detection, the binding affinities of the different Ru(II)(bpy)$_3$ complexes synthesised were assessed (Table 2.3). As can be seen Ru(II)(bpy)$_3$ complex 26 with 24 carboxylic acids has the highest binding affinity, the two Ru(II)(bpy)$_3$ complexes 31 and 32 with 12 carboxylic acids have lower affinity, but similar affinities to each other, the two Ru(II)(bpy)$_3$ complexes 29 and 30 with 6 carboxylic acids bind with much lower affinity, and the two negative controls 34 and 35 don't bind. This is as would be expected for binding to the
basic amino acid residues present on cyt c. The similarity between Ru(II)(bpy)$_3$ complexes 31 and 32 and complexes 29 and 30 show that the binding interaction is more dependent on the overall global charge located around the Ru(II)(bpy)$_3$ complex rather than small changes in location of the charge.

Table 2.3  Binding affinities for the different Ru(II)(bpy)$_3$ complexes synthesised to cyt c, 5 mM sodium phosphate, 0.2 mg mL$^{-1}$ BSA, pH 7.5

<table>
<thead>
<tr>
<th>Ru(II)(bpy)$_3$ complex</th>
<th>$K_a/ \mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>29</td>
<td>114 ± 20</td>
</tr>
<tr>
<td>30</td>
<td>65.9 ± 7.0</td>
</tr>
<tr>
<td>31</td>
<td>2.58 ± 0.72</td>
</tr>
<tr>
<td>32</td>
<td>1.73 ± 0.50</td>
</tr>
<tr>
<td>26</td>
<td>0.0429 ± 0.0031</td>
</tr>
<tr>
<td>34</td>
<td>&gt;100</td>
</tr>
<tr>
<td>35</td>
<td>&gt;&gt;100</td>
</tr>
</tbody>
</table>

2.4.1  UV/Vis ascorbate reduction assay

Figure 2.6  Ascorbate (0.75 mM) reduction of cyt c (16 μM) in the presence of various Ru(II)(bpy)$_3$ complexes (16 μM), measured by change in absorbance at 550 nm over time, 5 mM sodium phosphate, pH 7.4

An orthogonal assay to detect binding of these Ru(II)(bpy)$_3$ complexes to cyt c is to monitor the rate of ascorbate reduction of cyt c and see how the Ru(II)(bpy)$_3$ complexes affect this rate. In an oxidising environment the iron in the haem of cyt c exists as Fe(III), however it can be reduced to Fe(II) by addition of ascorbate. This reduction of the iron is accompanied with the appearance of a UV/Vis absorbance peak at 550 nm. The rate of
reduction can thus be seen using a UV/Vis spectrometer by measuring the absorption at 550 nm over time. The binding of a molecule to the surface of cyt c, close to the haem group, will block the approach of ascorbate and thus reduce the rate of ascorbate reduction.

This assay was performed for all the Ru(II)(bpy)$_3$ complexes (Figure 2.6) and, as can be seen, this mirrors the luminescence quenching assay results, whereby the most acidic Ru(II)(bpy)$_3$ complex 26 reduces the rate of cyt c reduction the most, and the Ru(II)(bpy)$_3$ complexes 31 and 32 reduce the rate less, but by similar amounts. The other Ru(II)(bpy)$_3$ complexes don’t reduce the rate of ascorbate reduction, indicating that they aren’t binding over the haem exposed edge or are binding with low affinity, as expected.

2.5 Cyt c/CCP PPI inhibition

The work presented in this section was performed by Dr. Maria Filby, aided by Dr. Michael Webb.

Previously these Ru(II)(bpy)$_3$ complexes had been hypothesised to bind at the haem exposed edge of cyt c, due to the location of the basic amino acid residues and the reduced rate of cyt c reduction by ascorbate. The haem-exposed edge is the location of the CCP binding site, therefore the Ru(II)(bpy)$_3$ complexes were hypothesised to inhibit the PPI.

Figure 2.7 Cyt c/CCP PPI inhibition by Ru(II)(bpy)$_3$ complex 26, a) Fluorescence spectrum of zinc substituted CCP alone (purple), with 1 eq. cyt c (red), with 1 eq. cyt c and 2 eq. Ru(II)(bpy)$_3$ complex 26 (green) and 2 eq. of Ru(II)(bpy)$_3$ complex 26 alone, b) Native agarose gel, showing the migration of cyt c, CCP and Ru(II)(bpy)$_3$ complexes

In order to test this hypothesis the fluorescence spectra of Zn-substituted CCP was taken alone, and when bound to cyt c (Figure 2.7a). Upon addition of cyt c, the fluorescence band of CCP at ~580 nm was quenched. On addition of Ru(II)(bpy)$_3$ complex 26 to this cyt c/CCP complex the fluorescence band at 580 nm was restored, along with
giving the appearance of the Ru(II)(bpy)$_3$ complex 26 luminescence emission band at ~635 nm. This luminescence band at ~635 nm is however quenched compared to the Ru(II)(bpy)$_3$ complex 26 alone, showing it as bound to cyt c. This indicated that the Ru(II)(bpy)$_3$ complex 26 displaced cyt c from CCP, thus inhibiting the PPI.

A native agarose gel was also performed, to corroborate these findings. Cyt c and CCP migrate towards the cathode and anode respectively, but when they complex together this migration was retarded. The presence of Ru(II)(bpy)$_3$ complex 26 retarded the migration of cyt c to the cathode, but the CCP is less affected (Lane 5). Thus, again, indicating the Ru(II)(bpy)$_3$ complex 26 binds to cyt c, inhibiting the cyt c/CCP interaction.

### 2.6 Binding in different conditions

Previously protein surface mimetics, including Ru(II)(bpy)$_3$ complexes, have been designed for binding to cyt c with charge-charge complementarity in mind, designing scaffolds functionalised with carboxylic acids in order to bind to the basic amino acid residues on cyt c. However little information has been obtained as to how these molecules bind to cyt c, and how this compares to its native protein partners, and thus if they are indeed mimicking the recognition of a native PPI, cyt c/CCP.

To find out more information as to how these molecules interact with cyt c, binding of the Ru(II)(bpy)$_3$ complexes 26 and 31 was tested in a variety of different conditions, using the previously described luminescence quenching. The effects of different conditions on cyt c binding of these Ru(II)(bpy)$_3$ complexes were compared to the binding of CCP to cyt c, allowing an understanding of whether the binding of the Ru(II)(bpy)$_3$ complex to cyt c indeed mimics this native PPI, to be developed.

#### 2.6.1 Temperature

Binding of Ru(II)(bpy)$_3$ complexes 26 and 31, using the luminescence quenching assay, was tested at different temperatures. This allows a van’t Hoff analysis to be performed (Eq. 2.3) to determine the thermodynamic parameters of binding. The van’t Hoff equation is derived from the Gibbs free energy definition (Eq. 2.1) and the Gibbs free energy isotherm equations (Eq. 2.2), where $\Delta H$ and $\Delta S$ are assumed to be temperature independent.

\[
\Delta G = \Delta H - T\Delta S \\
\Delta G = -RT\ln K
\]  

Eq. 2.1  
Eq. 2.2
\[ \ln K = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \]  
Eq. 2.3

\( \Delta G = \) change in Gibb’s free energy, \( \Delta H = \) change in enthalpy, \( \Delta S = \) change in entropy, \( T = \) temperature (in Kelvin), \( R = \) gas constant, \( K = \) binding constant

\[ \text{Figure 2.8} \] van’t Hoff analysis for the binding of \( \text{Ru(II)(bpy)}_3 \) complexes 26 and 31 measured in 5 mM sodium phosphate, 0.2 mg mL\(^{-1}\) BSA, pH 7.5

The binding of the two \( \text{Ru(II)(bpy)}_3 \) complexes do fit the van’t Hoff equation (Figure 2.8), allowing the thermodynamic parameters (\( \Delta H, \Delta S \) and \( \Delta G \)) to be derived (Table 2.4). The thermodynamic parameters for the two \( \text{Ru(II)(bpy)}_3 \) complexes were then compared to the native PPI to see if and how the \( \text{Ru(II)(bpy)}_3 \) complex/cyt c interaction mimics the native cyt c/CCP PPI.

\[ \text{Table 2.4} \] Thermodynamic parameters derived from the van’t Hoff analysis for the binding of the \( \text{Ru(II)(bpy)}_3 \) complexes 26 and 31 to cyt c in 5 mM sodium phosphate, 0.2 mg mL\(^{-1}\) BSA, pH 7.5, errors derived from triplicate experiments, and literature values for the cyt c/CCP interaction in similar conditions (10 mM potassium phosphate, pH 6.0, by ITC at 25 °C)\(^{55}\)

<table>
<thead>
<tr>
<th>( \Delta H / \text{kJ mol}^{-1} )</th>
<th>31</th>
<th>26</th>
<th>CCP(^{55})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T \Delta S (25 \degree \text{C}) / \text{kJ mol}^{-1} )</td>
<td>24.5 ( \pm ) 0.4</td>
<td>16.0 ( \pm ) 3.0</td>
<td>38.4 ( \pm ) 0.9</td>
</tr>
<tr>
<td>( \Delta G (25 \degree \text{C}) / \text{kJ mol}^{-1} )</td>
<td>-31.0 ( \pm ) 0.4</td>
<td>-42.3 ( \pm ) 0.0</td>
<td>-27.9 ( \pm ) 1.0</td>
</tr>
</tbody>
</table>
The binding of Ru(II)(bpy)$_3$ complex 31 to cyt c is predominantly entropically driven whereas the binding of Ru(II)(bpy)$_3$ complex 26 is both entropically and enthalpically driven. In comparison, the binding of the native cyt c/CCP PPI is entropically controlled and is even mildly enthalpically unfavourable. Thus, Ru(II)(bpy)$_3$ complex 31, with fewer carboxylates, is a closer match to cyt c’s interaction with its endogenous protein partner, CCP, showing this Ru(II)(bpy)$_3$ complex is indeed acting as a protein surface mimic. The enhanced binding of Ru(II)(bpy)$_3$ complex 26, is likely derived from the additional carboxylic acids forming increased numbers of salt bridges with the basic amino acids on the cyt c surface, increasing the enthalpic contribution to binding.

2.6.2 Ionic strength

To further understand the electrostatic contribution to binding, the binding affinities of Ru(II)(bpy)$_3$ complexes 26 and 31 were determined at different ionic strengths (I). The binding of both Ru(II)(bpy)$_3$ complexes 26 and 31 is highly dependent upon ionic strength (Table 2.5) with binding affinity decreasing with increasing ionic strength, suggesting electrostatic contributions dominate the binding of both of these Ru(II)(bpy)$_3$ complexes to cyt c, as would be expected. The binding of CCP to cyt c is also highly dependent on ionic strength, indicating again that the Ru(II)(bpy)$_3$ complex/cyt c interaction is mimicking that of the native cyt c/CCP PPI.

Table 2.5 Binding of Ru(II)(bpy)$_3$ complexes 26 and 31 in variable ionic strengths, 5 mM sodium phosphate, 0.2 mg mL$^{-1}$ BSA, pH 7.5, variable concentration NaCl, n.d. = not determined

<table>
<thead>
<tr>
<th>Ionic strength/ mM</th>
<th>31 $K_d$ / μM</th>
<th>26 $K_d$ / nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.39</td>
<td>2.88 ± 0.46</td>
<td>25.3 ± 2.4</td>
</tr>
<tr>
<td>13.39</td>
<td>4.25 ± 0.47</td>
<td>64.8 ± 13.7</td>
</tr>
<tr>
<td>18.39</td>
<td>10.30 ± 1.61</td>
<td>196.5 ± 59.2</td>
</tr>
<tr>
<td>28.39</td>
<td>20.23 ± 0.16</td>
<td>426.5 ± 59.8</td>
</tr>
<tr>
<td>48.39</td>
<td>n.d.</td>
<td>2040.9 ± 152.6</td>
</tr>
</tbody>
</table>

The $K_d$ values were fit to the Debye-Hückel relationship (Eq. 2.4), (the theory behind and derivation of this is presented in the Appendix I) (Figure 2.9) in this case using a Güntelberg approximation (Eq. 2.5), which is valid up to $I = 100$ mM.

$$
\log K_d = \log K_d^0 - 0.509Z_1Z_2\mu
$$

Eq. 2.4
$$\mu \approx \frac{1}{\sqrt{1 + \sqrt{I}}}$$  \hspace{1cm} \text{Eq. 2.5}

$K_d = \text{dissociation constant}, \ K_d^0 = \text{theoretical dissociation constant at 0 ionic strength}, \ Z_1 \ \text{and} \ Z_2 = \text{charges on species 1 and 2}, \ \mu = \text{a function of the ionic strength (empirically derived)}, \ I = \text{ionic strength}$

From this relationship the parameters $K_d^0$ and $Z_1Z_2$ can be established, providing an estimate of the binding affinity at $I = 0$ and the product of the interacting positive and negative charges, respectively. The data were consistent with the Güntelberg approximation for both Ru(II)(bpy)$_3$ complexes (Figure 2.9), giving a linear relationship. The calculated values of $K_d^0$ (Table 2.6) show high affinity binding for Ru(II)(bpy)$_3$ complex 26 and weaker binding for Ru(II)(bpy)$_3$ complex 31 at zero ionic strength, with the value for CCP being between these values.

![Figure 2.9 Debye-Hückel analysis for the binding of Ru(II)(bpy)$_3$ complexes 26 and 31 to cyt c and literature values for CCP for comparison, measured in 5 mM sodium phosphate, variable concentration NaCl, 0.2 mg mL$^{-1}$ BSA, pH 7.5](image)

<table>
<thead>
<tr>
<th></th>
<th>31</th>
<th>26</th>
<th>CCP$^{154}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d^0$ / nM</td>
<td>253 ± 5</td>
<td>1.11 ± 0.21</td>
<td>40.7 ± 23.0</td>
</tr>
<tr>
<td>$Z_1Z_2$</td>
<td>25.9 ± 1.9</td>
<td>35.6 ± 1.3</td>
<td>28.8 ± 4.8</td>
</tr>
</tbody>
</table>

Table 2.6 Values derived from the Debye-Hückel approximation for Ru(II)(bpy)$_3$ complexes 26 and 31 and CCP$^{154}$
The $Z_1Z_2$ value provides an indication of the charges involved in the interaction, with Ru(II)(bpy)$_3$ complex 26 having a larger value than Ru(II)(bpy)$_3$ complex 31 and CCP. Using these data, the charge on the Ru(II)(bpy)$_3$ complex interacting with cyt $c$ can be estimated. Assuming cyt $c$ has the same charge in all cases (calculated to be $\sim 6$ at pH 7.5), the charge on Ru(II)(bpy)$_3$ complex 26 and 31 and CCP can be calculated to be 5.9, 4.3 and 4.8, respectively. Ru(II)(bpy)$_3$ complex 31 and CCP have relatively similar charges, indicating similar interactions with cyt $c$, further showing that its binding to cyt $c$ is mimicking that of the native PPI. Ru(II)(bpy)$_3$ complex 26 has a larger charge, indicating increased electrostatic interactions with cyt $c$, showing that it is possible to increase the interactions in a native PPI, in order to gain high affinity protein surface ligands. Accounting for the crudeness of the Debye-Hückel approximation where small ($\sim 3$ Å), evenly dispersed charges are assumed (even when using the Güntelberg extension, which extends past the single point charges used in the first (Debye-Hückel) approximation), the data indicate that perhaps not all the carboxylates are deprotonated under the assay conditions (pH 7.5) and/or that a limited number of the carboxylates are needed for the protein surface recognition, even fewer than the 4 isophthalate arms as found by the Ohkanda group using heteroleptic complexes.

2.6.3 Binding in different buffers

![Buffers](image)

**Figure 2.10** Buffers i) Structures of the buffer components, ii) Plausible interactions of the buffer components with the lysine residues on cyt $c$, and Tris/btp with carboxylates on the Ru(II)(bpy)$_3$ complex 26

The binding in different buffers can also give a further indication as to what interactions are important for binding, as for binding to occur, negatively charged anions must be displaced from the positively charged surface of cyt $c$, and positively charged cations must be displaced from the negatively charged surfaces of the Ru(II)(bpy)$_3$ complex.
complexes. The binding affinity of Ru(II)(bpy)$_3$ complex 26 to cyt c was tested in a range of different buffers (Table 2.7), with different structures and potential interactions with cyt c and Ru(II)(bpy)$_3$ complex 26 possible (Figure 2.10).

Table 2.7 Cyt c binding affinities of Ru(II)(bpy)$_3$ complex 26 in a range of different buffers, all buffers at 5 mM, pH 7.5 with 0.2 mg mL$^{-1}$ BSA

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$K_a$ / nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate</td>
<td>42.9 ± 3.1</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>26.2 ± 3.1</td>
</tr>
<tr>
<td>MOPS</td>
<td>35.2 ± 3.1</td>
</tr>
<tr>
<td>HEPES</td>
<td>31.2 ± 3.1</td>
</tr>
<tr>
<td>Tris</td>
<td>106.3 ± 32.6</td>
</tr>
<tr>
<td>Bis-tris propane (btp)</td>
<td>133.5 ± 37.4</td>
</tr>
</tbody>
</table>

There is little difference in the binding affinity between Ru(II)(bpy)$_3$ complex 26 and cyt c in sodium and potassium phosphate buffer, indicating the interactions of the cationic buffer components with the Ru(II)(bpy)$_3$ complex 26 are not significant. There also is not a significant difference between the binding in the phosphate buffers and the sulfonic acid buffers (MOPS and HEPES), suggesting that the nature of the anion, and the hydrophobicity of the buffer are not significant. This reinforces the conclusions of the Debye-Hückel analysis, with the interaction being dominated by electrostatic contributions. For the tris buffers (btp and tris) a small decrease in binding affinity is seen compared to the other buffers; this is likely due to different interactions between the buffer and cyt c, and Ru(II)(bpy)$_3$ complex 26 and its chloride counterion. Both the ammonium and hydroxyl functionalities of the tris buffers may interact with cyt c and the Ru(II)(bpy)$_3$ complex 26 with the potential for chelating hydrogen bonding interactions (Figure 2.10b), this would diminish binding affinity, by masking both the carboxylic acid functionality on the Ru(II)(bpy)$_3$ complex as well as the lysine residues on cyt c.

2.6.4 pH

Cyt c is a stable protein that does not unfold over a wide range of pHs, however its ionisation state is affected by pH (Figure 2.11b), hence the pH of the solution is expected to affect the interaction of cyt c with the Ru(II)(bpy)$_3$ complexes, especially as the interaction is driven by electrostatic interactions. The effect of pH on the binding
affinity of Ru(II)(bpy)_3 complex 26 to cyt c was investigated. btp was used for this study as it allows for a broad pH range (pH 6.5 - 9.5) to be used in the same buffer.

The binding affinity follows an inverted bell shaped profile (Figure 2.11a), which maps well onto the ionisation state of cyt c (Figure 2.11b). The binding affinity is relatively constant between pH 7.0 and pH 8.5, with decreased binding affinity observed at pH 6.5 and pH 9.0. This again indicates electrostatics as a major contributor to the binding between the Ru(II)(bpy)_3 complex and cyt c.

Figure 2.11 Effect of pH on the binding of Ru(II)(bpy)_3 complex 26 to cyt c. a) Binding affinity over the range pH 6.5 – 9.0 (5 mM btp, 0.2 mg mL\(^{-1}\) BSA, variable pH), b) the electrostatic interaction factor (ω) of cyt c over a range of pHs (base limb of titration curve)\(^{159}\), c) Cyt c structure (PDB ID 1HRC)\(^{160}\) with residues that become protonated/deprotonated at pH 6.5 and 9.0, His-33 (pink) and Lys-79 (green) respectively

From these data, it is also possible to glean information as to a potential binding site, as at specific pHs different amino acids become protonated or deprotonated, and thus are changing their role in the binding interaction. The amino acid residues that become protonated/deprotonated at pH 6.5 and 9.0 are His-33 and Lys-79 respectively (Figure 2.11c).\(^{159}\) Lys-79 is located on the haem-exposed edge, where binding of Ru(II)(bpy)_3 complex 26 is hypothesised to occur, whereas His-33 is on the distal face of cyt c. There are a number of different reasons as to the decrease in binding at this pH despite hypothesised binding at a different site: i) Ru(II)(bpy)_3 complex 26 binds to different or multiple sites on cyt c, ii) the protonation of His-33 causes a small conformational change in the cyt c structure, affecting the binding interactions on the haem-exposed edge, iii) the protonation state of Ru(II)(bpy)_3 complex 26 is changed at pH 6.5, affecting its binding interaction. Upon closer inspection of the pH/K\(_d\) and pH/ionisation state relationships,
there is a slight discrepancy with the cyt c ionisation state dropping at pH 8.0 rather than pH 8.5, where the binding affinity diminishes, whereas this difference does not exist at the lower pH, with both binding affinity and ionisation state decreasing at pH 6.5. This implies that the binding of Ru(II)(bpy)₃ complex 26 masks Lys-79 and increases its pKₐ, whereas the His-33 protonation state is not affected by the binding of Ru(II)(bpy)₃ complex 26. Therefore the loss in binding affinity observed at pH 6.5 is likely to arise from changes in ionisation state of the Ru(II)(bpy)₃ complex 26, rather than that of His-33.

2.6.5 Conclusions of conditions screen

From the data obtained from screening the cyt c binding under different conditions it is possible to draw some conclusions as to how the Ru(II)(bpy)₃ complexes 26 and 31 bind to cyt c. The binding for both Ru(II)(bpy)₃ complexes is electrostatically driven, with the binding of Ru(II)(bpy)₃ complex 31 being entropically favourable and that of Ru(II)(bpy)₃ complex 26 being both entropically and enthalpically favourable. Compared to the native cyt c/CCP interaction, an entropy controlled, electrostatic interaction, the binding of Ru(II)(bpy)₃ complex 31 acts as a closer mimic. Increased enthalpic contributions for the binding of Ru(II)(bpy)₃ complex 26 arise from increased electrostatic interactions due to an increased number of carboxylates, allowing for further interactions over that in the native PPI. This shows it may be possible to use information on the binding interface of a known PPI, and enhance the interactions present when designing a molecular ligand in order to gain a high affinity protein surface mimetic.

2.7 NMR spectroscopy

From the UV/Vis ascorbate reduction data (2.4.1) it can be established that the Ru(II)(bpy)₃ complexes bind close to the haem group on cyt c, as otherwise there would be no reduction in the rate of cyt c reduction. Similarly, the pH profile data indicate binding at the haem-exposed edge, close to Lys-79. However, detailed information as to the binding site of these Ru(II)(bpy)₃ complexes had not been previously established. In order to study where these Ru(II)(bpy)₃ complexes are binding and to gain a fuller picture of the binding interaction protein NMR spectroscopy was performed. In this case a ¹H-¹⁵N HSQC was used, where the backbone amide bond N-H cross-correlations are monitored, and the spectrum of cyt c with and without the Ru(II)(bpy)₃ complex present compared. The shifting of cross-peaks on addition of the Ru(II)(bpy)₃ complex suggest binding close to the amino acid residue corresponding to that cross-peak, whereas if the cross-peak does not shift it indicates the Ru(II)(bpy)₃ complex binds at a distal site to that residue.
Ordinarily when performing protein NMR spectroscopy, a protein labelled with NMR-active $^{15}$N and/or $^{13}$C isotopes is required, dependent on the experiment being performed. The Astbury Centre had recently acquired a new 950 MHz NMR spectrometer so it was decided to see if this machine could detect naturally abundant levels of $^{15}$N in an HSQC experiment, both as a test for the instrument and to see if it was possible to detect binding of the Ru(II)(bpy)$_3$ complexes to cyt c. Cyt c is a good test for this capability as it can be bought in large (gram) quantities, and studied in high (millimolar) concentration solutions, as is required for the detection of the low natural abundance of $^{15}$N. NMR spectra were obtained with the help of Dr. Lars Kuhn and Dr. Arnout Kalverda.

### 2.7.1 Oxidised/reduced cyt c

One problem that could be observed in the NMR spectrum of cyt c is due to the presence of the haem iron. In the oxidised Fe(III) state present in normal, oxygen-rich, conditions the Fe(III) has a $d^5$ high spin electronic configuration; this has unpaired electrons, thus making it paramagnetic. Paramagnetism generates a local magnetic field, which leads to interference in the NMR spectrum. In the case of a protein NMR spectra this leads to line broadening of the resonances for atoms close to the paramagnetic atom. As binding was expected to be on the haem exposed edge, by the haem iron, it was thought that this may complicate the NMR spectrum in the region of interest. Therefore the Fe(III) was reduced to Fe(II) in order to run the NMR spectroscopy experiment. The simplest way to do this is to add sodium ascorbate to the buffer.

As a different buffer and oxidation state of cyt c was going to be used in the NMR spectroscopy experiment, the binding of Ru(II)(bpy)$_3$ complex 26 to cyt c under the reducing conditions was tested. A comparison was also made between chemically oxidised cyt c and reduced cyt c in the same buffer in order to see the effect cyt c reduction has on the binding affinity. Irreversibly, chemically oxidised cyt c was obtained by addition of $K_3$Fe(CN)$_6$.

**Table 2.8** Binding constants for Ru(II)(bpy)$_3$ complex 26 binding to oxidised and reduced cyt c (5 mM sodium phosphate, 0.1 mM sodium ascorbate, 0.2 mg mL$^{-1}$, pH 7.5), and the native PPI for comparison (3.5 mM potassium phosphate, pH 7.5)

<table>
<thead>
<tr>
<th></th>
<th>$K_a$ / nM</th>
<th>$CCP^{15}$ $K_a$ / nM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidised, Fe$^{3+}$</strong></td>
<td>49.6 ± 13.3</td>
<td>440 ± 110</td>
</tr>
<tr>
<td><strong>Reduced, Fe$^{2+}$</strong></td>
<td>92.4 ± 5.5</td>
<td>690 ± 200</td>
</tr>
</tbody>
</table>
The luminescence quenching assay was used to test the binding of Ru(II)(bpy)$_3$ complex 26 to the oxidised and reduced cyt $c$. In this case, a lower concentration sodium ascorbate buffer than was to be used in the NMR spectroscopy (0.1 mM rather than 2 mM) was employed due to the concentration of oxidised cyt $c$ stock being relatively low (0.508 mM), as a result of dilution during dialysis. As it is known that at high ionic strength the binding affinity is relatively weak it was decided to assess this binding in a lower ionic strength buffer to allow for the use of this cyt $c$ stock. As can be seen in Table 2.8 the binding affinity is similar between both the oxidised and reduced cyt $c$, as it is with the native PPI. This means that using the reduced cyt $c$ to obtain the NMR spectrum is a valid approach.

2.7.2 $^1$H-$^{15}$N HSQC of cyt $c$ alone

![Figure 2.12](image)

**Figure 2.12** NMR spectra of cyt $c$ alone a) 1-D $^1$H spectrum, b) Natural abundance $^1$H-$^{15}$N HSQC spectrum, both 2 mM cyt $c$, in 5 mM sodium phosphate, 2 mM sodium ascorbate, 10 % D$_2$O, pH 7.3 buffer

Following a promising $^1$H 1-D spectrum (Figure 2.12a) of 2 mM cyt $c$ in the 5 mM sodium phosphate, 2 mM sodium ascorbate, 10 % D$_2$O, pH 7.3 buffer, a $^1$H-$^{15}$N HSQC was obtained. This gave clearly defined cross-peaks (Figure 2.12b) which could be assigned using a previously reported HSQC assignment. This shows the utility of the 950 MHz NMR spectrometer, showing that it is possible to obtain good, assignable $^1$H-$^{15}$N HSQC spectra using natural abundance $^{15}$N.

2.7.3 $^1$H-$^{15}$N HSQC spectrum of Ru(II)(bpy)$_3$ complex 31 with cyt $c$

Having obtained a good spectrum for cyt $c$ alone, a $^1$H-$^{15}$N HSQC spectrum of 1 mM cyt $c$ with 0.5 mM Ru(II)(bpy)$_3$ complex 31 was obtained (Figure 2.13). This spectrum could similarly be assigned, with some cross-peaks having stayed in the same place, others having shift changes ranging from 0.015 - 0.05 ppm and others disappearing. This
**Figure 2.13** $^1$H-$^{15}$N HSQC NMR data of Ru(II)(bpy)$_3$ complex 31 binding to cyt c. a) Region of the overlaid $^1$H-$^{15}$N HSQC spectrum of cyt c (red) and cyt c with 0.5 eq Ru(II)(bpy)$_3$ complex 31 (blue). Inset shows zoom in of part of the spectrum, showing some cross-peaks staying the same, some having shifted and one disappearing. b) $^1$H-$^{15}$N chemical shift differences ($\Delta\delta$) for the different amino acid residues with and without Ru(II)(bpy)$_3$ complex 31. Gaps are for prolines, unassigned amino acids, red peaks are amino acids for which the signal disappears (arbitrarily $\Delta\delta$ shown to be 0.05) - due to significant line-broadening of NH cross-peaks - on addition of Ru(II)(bpy)$_3$ complex 31. c) Chemical shift perturbation map of cyt c, molecular surface of cyt c generated from PyMol (PDB ID 1U75), with colouring corresponding to the extent of chemical shift changes ($\Delta\delta$) on addition of the Ru(II)(bpy)$_3$ complex 31. Amino acid residues with $^1$H-$^{15}$N resonances that disappear in dark red, exhibit large chemical shift changes ($\Delta\delta>0.03$) in red, moderate changes ($\Delta\delta>0.02$) in orange, small changes ($\Delta\delta>0.015$) in yellow-orange and very small chemical shift changes ($\Delta\delta>0.01$) in yellow. d) Perturbation map of cyt c (as in c), corresponding to the top central structure) in complex with CCP (purple), (PDB ID 1U75). For the presence of protein-ligand interactions. These chemical shift changes were mapped onto the structure of cyt c where they indicate binding to one side of the haem group of cyt c, with the opposite face having very few amino acid residues with sizeable shifts in their HSQC cross-peaks (**Figure 2.13c**), indicating a binding site to one side of the haem exposed edge. The binding site identified here is in a similar location to that of carboxylate functionalised porphyrins, as determined by the Crowley group. Mapping these amino acid residues onto the cyt c/CCP PPI structure (**Figure 2.13d**) it can be seen
that the amino acid residues whose cross-peaks have shifted are in and around the cyt c/CCP PPI interface, indicating Ru(II)(bpy)$_3$ complex 31 binds in the same region as CCP, and so indeed could be mimicking this PPI.

2.7.4 $^1$H-$^{15}$N HSQC spectrum of Ru(II)(bpy)$_3$ complex 26 with cyt c

![Figure 2.14](image)

Figure 2.14 NMR spectra for the binding of Ru(II)(bpy)$_3$ complex 26 to cyt c a) 1-D $^1$H NMR spectrum of cyt c alone (black), cyt c with 0.5 eq Ru(II)(bpy)$_3$ complex 31 (blue) and with 0.5 eq Ru(II)(bpy)$_3$ complex 26 (green), b) $^1$H-$^{15}$N chemical shift differences ($\Delta\delta$) for the different amino acid residues with and without Ru(II)(bpy)$_3$ complex 26. Gaps are for prolines, unassigned amino acids, and signals that disappear, c) Chemical shift perturbation map of cyt c with Ru(II)(bpy)$_3$ complex 26, molecular surface of cyt c generated as in Figure 2.13, except showing amino acid residues whose cross-peaks disappear in blue. This view is of the binding site of Ru(II)(bpy)$_3$ complex 31

Attempts were also made to obtain spectra with both 0.5 and 1 equivalents of Ru(II)(bpy)$_3$ complex 26. On obtaining a 1-D $^1$H NMR spectrum (Figure 2.14a) (green) it could be seen that the peaks have broadened compared to both the cyt c alone (black) and with 0.5 equivalents of Ru(II)(bpy)$_3$ complex 31 (blue). This could indicate the formation of a larger species, for example many Ru(II)(bpy)$_3$ complexes 26 binding to cyt c or the Ru(II)(bpy)$_3$ complex(es) 26 causing oligomerisation of cyt c. This is perhaps unsurprising as carboxylate functionalised porphyrins have been shown to have multiple binding sites
on cyt c, and to multimerise cyt c at high (millimolar) concentrations.\textsuperscript{103} Upon attempting a \textsuperscript{1}H-\textsuperscript{15}N HSQC of cyt c with 0.5 equivalents of Ru(II)(bpy)\textsubscript{3} complex 26, only 41\% of the cross-peaks (Figure 2.14b) present in the cyt c alone were present in the spectrum, compared to 93\% with 0.5 eq. of Ru(II)(bpy)\textsubscript{3} complex 31. This means that detailed information as to the binding site could not be gleaned, for example a view of the Ru(II)(bpy)\textsubscript{3} complex 31 binding site is shown in Figure 2.14c, with the shifts for Ru(II)(bpy)\textsubscript{3} complex 26 shown as varying colours dependent on shift difference, and blue indicating signals that have disappeared. This again indicates the formation of larger species, or potentially that the structure of the cyt c is disrupted, however previous studies from the Wilson group have shown that Ru(II)(bpy)\textsubscript{3} complex 26 does destabilise cyt c but does not change the structure at 20 °C, the temperature at which the NMR spectroscopy was run.\textsuperscript{144}

\subsection*{2.7.5 \textit{S. cerevisiae} cyt c binding}

![Figure 2.15](image)

**Figure 2.15** Binding isotherms for a) Ru(II)(bpy)\textsubscript{3} complex 31 and b) Ru(II)(bpy)\textsubscript{3} complex 26 with cyt c from \textit{S. cerevisiae}

Further evidence for multiple binding sites for Ru(II)(bpy)\textsubscript{3} complex 26 on cyt c has been obtained by looking at the binding of Ru(II)(bpy)\textsubscript{3} complex 26 to cyt c from a different species. So far all the binding discussed has been with horse heart cyt c, with data fitting to a 1:1 binding isotherm. On testing the binding of Ru(II)(bpy)\textsubscript{3} complex 31 with \textit{S. cerevisiae} (yeast) cyt c, a similar binding isotherm (Figure 2.15a) with similar $K_d$ (2.81 ± 0.68 \textmu M compared to 2.58 ± 0.72 \textmu M with horse heart cyt c) was obtained, as would be expected, especially for such an evolutionarily conserved protein. However, looking at the binding of Ru(II)(bpy)\textsubscript{3} complex 26 to the \textit{S. cerevisiae} cyt c, a different shaped binding isotherm was obtained (Figure 2.15b). This can be hypothesised to be due to a second binding event, and is consistent with the NMR data with such broadening of peaks all over the protein.
2.8 Conclusions

The synthesis and cyt c binding of 7 different Ru(II)(bpy)₃ complexes has been established. A luminescence quenching assay was used to show that increasing numbers of carboxylates correlate with increased cyt c binding affinity. A UV/Vis cyt c reduction assay corroborated these result. The binding of two of these Ru(II)(bpy)₃ complexes 26 and 31 was assessed in varying conditions, the effect of these differing conditions was compared to that with one of cyt c's native protein partners, CCP, for which Ru(II)(bpy)₃ complex 26 had been shown to inhibit the PPI. This showed that the smaller Ru(II)(bpy)₃ complex 31 bound in a similar manner to CCP with binding being an electrostatic, entropy driven process, whereas the binding of the larger Ru(II)(bpy)₃ complex 26 was found to be both entropy and enthalpy driven with the increased enthalpic contributions arising from increased numbers of electrostatic interactions. Natural abundance ¹H-¹⁵N HSQC NMR spectra were obtained for both cyt c alone and Ru(II)(bpy)₃ complex 31 bound to cyt c, showing binding occurring at the cyt c/CCP binding interface, and indicating that the binding of Ru(II)(bpy)₃ complex 31 to cyt c does indeed mimic that of CCP. This shows that using known information about a native PPI it is possible to mimic the PPI in order to achieve ligands for one of the protein partners. The enhanced binding of Ru(II)(bpy)₃ complex 26, shows it is possible to enhance the known binding interactions present in the native PPI to achieve high affinity ligands for one protein partner. This knowledge could be used for the design of new, high affinity, ligands for known PPIs of therapeutic interest.
3 Design of multivalent Ru(II)(bpy)₃ and porphyrin scaffolds for dynamic combinatorial chemistry

Dynamic combinatorial chemistry (DCC) is an approach whereby a thermodynamic equilibrium of many competing reversible chemical reactions is generated, which can be exploited for receptor discovery. The equilibrium is established, in the presence and absence of a template, in order to determine which compounds out of the dynamic combinatorial library (DCL) generated are amplified and therefore bind to that template (Figure 3.1).¹⁶²-¹⁶⁵ If a protein template is used, ligands for that protein template may be generated, with the highest affinity ligands being amplified, in the presence of the protein, compared to in its absence. This allows for the selection of high affinity ligands from an array of potential candidates.

Figure 3.1 Cartoon schematic of DCC around a multivalent scaffold. A DCC scaffold is incubated with different groups that can reversibly covalently attach around the scaffold under thermodynamic equilibrium conditions. This thermodynamic equilibrium is reached in the absence and presence of a template protein; the molecules that bind to this template become enriched in the presence of the template compared to without, as they become more thermodynamically stable.

A major limitation in the identification of protein surface mimetics, is the generation of large multivalent compounds with differing binding groups projected around the surface. DCC could allow the reversible sampling of many different groups for their protein binding affinity around the protein surface mimetic scaffold, thus allowing the development of structurally and compositionally diverse protein surface mimetics. To this end, the design of Ru(II)(bpy)₃ complex and tetraphenyl-porphyrin DCC scaffolds has been attempted, allowing for the potential use of two different protein surface mimetic scaffolds in biologically relevant DCLs.
3.1 Biologically compatible DCC

As the aim of using these multivalent dynamic systems is to generate multivalent protein ligands, the system must be biologically compatible. For DCC to be biologically compatible, the reactions must be performed in an aqueous environment, at neutral pH, at an appropriate temperature and must reach equilibrium within a reasonable time frame.\textsuperscript{166}

<table>
<thead>
<tr>
<th>Reaction name</th>
<th>Reaction</th>
<th>Protein examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imine formation</td>
<td><img src="image" alt="Imine formation" /></td>
<td>SARS coronavirus</td>
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<tr>
<td></td>
<td></td>
<td>SARS-CoV-\textsuperscript{pro}</td>
</tr>
<tr>
<td>Hydrazine formation</td>
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<td>γ-amino butyric acid transporter</td>
</tr>
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<td></td>
<td>1\textsuperscript{67}</td>
</tr>
<tr>
<td>Acyl hydrazine formation</td>
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<td>Glutathione S-transferase\textsuperscript{168}</td>
</tr>
<tr>
<td>Hemithioacetal formation</td>
<td><img src="image" alt="Hemithioacetal formation" /></td>
<td>β-galactosidase\textsuperscript{169}</td>
</tr>
<tr>
<td>Thioether formation</td>
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<td>Concanavalin A\textsuperscript{171}</td>
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<tr>
<td>Boronate ester formation</td>
<td><img src="image" alt="Boronate ester formation" /></td>
<td>2-oxoglutarate dependent oxygenases\textsuperscript{172}</td>
</tr>
<tr>
<td>Metal ligand coordination</td>
<td><img src="image" alt="Metal ligand coordination" /></td>
<td>Lectins\textsuperscript{132} (Fe(II)-bpy)</td>
</tr>
</tbody>
</table>

Table 3.1 Examples of biologically compatible DCC reactions\textsuperscript{166}

Examples of biologically compatible DCC reactions, are shown in Table 3.1. Many of these use the same types of linkages with imines and various sulfur containing bonds being common.

DCLs have also been designed where the system is first equilibrated in a non-biologically relevant context, and the mixture of products obtained tested against the biological molecule of interest. The procedure can then be repeated with different components missing, to determine which components of the mixture contribute to
binding. This allows a wider range of reaction types to be used, but involves many more DCL experiments, and is not a truly adaptive system.

Previously, in the Wilson group, an Fe(II)(bpy)$_3$ system, akin to that used by the Sasaki and de Mendoza groups described in the Chapter 1,\textsuperscript{131,132} was developed,\textsuperscript{173} but this methodology did not prove to be reproducible. Therefore a different type of reversible reaction, was attempted around a Ru(II)(bpy)$_3$ core. The multivalent nature of the scaffolds mean that the use of thiol-based DCC would be problematic due to the potential for polymerisation of the scaffold by disulfide bond formation. Acyl hydrazones were chosen as they do not require a fixation reaction at the end of the DCL generation, as required, for example, for imine formation. Acyl hydrazides are also facile to synthesise from methyl esters, for which Ru(II)(bpy)$_3$ functionalised species have previously been synthesised.\textsuperscript{142}

3.2 Ru(II)(bpy)$_3$ scaffolds

3.2.1 Initial scaffold

\textbf{Scheme 3.1} Hypothesised initial DCC system

A hydrazide Ru(II)(bpy)$_3$ scaffold 43, initially directly attached to the Ru(II)(bpy)$_3$ core was chosen as a good starting point. This would allow for 6 different hydrazones to form around the central Ru(II)(bpy)$_3$ complex core (\textbf{Scheme 3.1}). It was hypothesised that conditions previously established for hydrazone exchange in biological media by the Greaney group could be used.\textsuperscript{168} Here the hydrazone exchange is performed in a biological buffer with aniline 45 acting as a nucleophlic catalyst, catalysing the hydrazone exchange at pH 6.2, as opposed to the acidic conditions generally required. This would allow the DCL to be incubated with a protein in order to select for a protein ligand.
3.2.1.1 Synthesis

Scheme 3.2 Synthesis of the initial Ru(II)(bpy)$_3$ complex hydrazide scaffold 43

Hydrazides can readily be formed by the reaction of hydrazine monohydrate with a methyl or ethyl ester. Initially this was attempted on the methyl ester ligand 47 (Scheme 3.2 top), however the hydrazide ligand 48 formed was only soluble in very strong acids, preventing further reaction to form the hydrazide Ru(II)(bpy)$_3$ complex 43. The hydrazide Ru(II)(bpy)$_3$ complex 43, however, could easily be formed from the methyl ester Ru(II)(bpy)$_3$ complex 49 (Scheme 3.2 bottom), by refluxing it with hydrazine monohydrate in methanol. This gave the hydrazide Ru(II)(bpy)$_3$ complex 43 as a methanol insoluble, but water-soluble solid, allowing it to be separated from the excess hydrazine. The hydrazide Ru(II)(bpy)$_3$ complex 43 was found to be oxygen sensitive, degrading over the period of a few hours, thus this compound could not be stored. Degradation of hydrazides with atmospheric oxygen has previously been reported, with first row transition metal cations increasing the rate of this reaction.$^{174}$

3.2.1.2 Hydrazone formation

Scheme 3.3 Hydrazone formation on initial hydrazide Ru(II)(bpy)$_3$ complex 43
Various hydrazone Ru(II)(bpy)_3 complexes could be formed from the hydrazide Ru(II)(bpy)_3 complex 43 (Scheme 3.3), with almost immediate reaction on addition of the appropriate aldehyde 44. Benzaldehyde derivatives were used as they lack α-protons and hence cannot form enamines, which could subsequently react with the excess aldehyde present. These hydrazone Ru(II)(bpy)_3 complexes 46 were stable, allowing the compound to be stored and used for analysis of the hydrazone exchange required for the generation of a DCL.

### 3.2.1.3 Hydrazone exchange

Prior to the generation of a complex DCL it was necessary to show that hydrazone exchange occurs. High resolution mass spectrometry (HRMS) was used to show this exchange, as it requires very little material and measurements can be taken at various time points from the same reaction mixture. In following these species by mass spectrometry an assumption that all species ionise equally so that their proportion in the mass spectrum is the same as that in the solution is made. In the case of the Ru(II)(bpy)_3 complexes, we can -to some extent- assume that the species ionise in similar proportions to their presence in the solution, as they exist as 2+ species and so are already ionised. The ionisation is therefore much less dependent on the ionisation susceptibility of different functional groups, as would be required of an organic molecule.

![Scheme 3.4 Radical cleavage of N-N bond in mass spectrometer](image.png)

Obtaining the mass spectrum of the various different hydrazone Ru(II)(bpy)_3 complexes 46 formed revealed the M^{2+} ion peak as expected, but also revealed peaks for radical degradation of the Ru(II)(bpy)_3 complex in the mass spectrometer, through cleavage of the N-N bond (Scheme 3.4). This ties in well with the instability of the hydrazide Ru(II)(bpy)_3 complex in oxygen as this also relies on the breaking of the N-N bond using an oxygen single electron. Knowledge of this, however, allows all peaks in the mass spectrum can be assigned.

A benzaldehyde hydrazone Ru(II)(bpy)_3 complex 46a was incubated with 100 equivalents (eq.) (16.7 eq. per hydrazone) of 2,4-dimethoxy benzaldehyde 45b, in order to see if hydrazone exchange could occur and on what time scale any hydrazone exchange occurs (Scheme 3.5). For initial tests an aniline 44 catalyst was used, as this was observed to catalyse the hydrazone exchange, by Greaney et al.168 This exchange was performed in
1:1 acetonitrile:water, as this gave good solubility of all species involved, and provided a test to see if the hydrazone exchange could indeed occur without the addition of an acid.

Scheme 3.5 Hydrazone exchange a) Reaction to be performed, b) Cartoon schematic of reaction, c) Cartoon schematic of individual hydrazone exchanges

As can be seen in Figure 3.2a, there was a change in the mass spectrum from time 0 and after 4 days, with many hydrazone exchanges having occurred. However complete hydrazone exchange, as would be expected for the addition of 100 eq. (over 15 equivalents per hydrazone) was not observed. Following this hydrazone exchange over a time course (Figure 3.2b) it can be seen that the initial species 46a (0) decreased over time and the species from one hydrazone exchange (1) at first increased, and then decreased. The species from subsequent hydrazone exchanges (2, 3, 4 etc.) increased over time at decreasing rates for later hydrazone exchanges. However, the system did not reach thermodynamic equilibrium after 32 hours, as this would result in the proportion of each species remaining constant (flat-lining), which was not observed. In terms of a biological system, the use of such a system would take too long, and the protein would potentially not be stable for this length of time. Incubation of the same system without a catalyst showed no hydrazone exchange occurring in the same time period, showing the requirement for the aniline 44 catalyst for hydrazone exchange to occur.
Figure 3.2 Hydrazide exchanges on the initial Ru(II)(bpy)$_3$ hydrazide scaffold 46. a) Mass spectra depicting the starting benzaldehyde hydrazide Ru(II)(bpy)$_3$ species 46a (100 μM) (top) and the species present after 4 days (bottom) of incubation with 2,4-dimethoxy benzaldehyde 44b (10 mM) with aniline 45 catalyst (10 mM) in 1:1 MeCN:H$_2$O. b) Time course following the species present at different time points

3.2.2 Ru(II)(bpy)$_3$ scaffold developments

The initial hydrazide Ru(II)(bpy)$_3$ complex scaffold 43 demonstrated that hydrazide exchange can be performed around a Ru(II)(bpy)$_3$ core. However, scaffold 43 was not well optimised, for two reasons: i) the hydrazide Ru(II)(bpy)$_3$ complexes 46 formed were not soluble in biologically relevant media (requiring high DMSO concentrations to solubilise in water), ii) the degradation by N-N bond cleavage in the mass spectrometer complicated any analysis of the kinetics of hydrazide exchange.
3.2.2.1 Glycine hydrazide Ru(II)(bpy)\textsubscript{3} complex

Scheme 3.6 Synthesis of glycine hydrazide and hydrazone Ru(II)(bpy)\textsubscript{3} complexes 49 and 50

The N-N bond cleavage in the mass spectrometer was hypothesised to be due to stabilisation of the radical formed by the bipyridine π-system attached to the ruthenium(II) centre. It was thought that this interaction could be broken by introducing an sp\textsuperscript{3} centre between the hydrazide and the Ru(II)(bpy)\textsubscript{3} core. In order to achieve this, an ethyl glycine complex 42f was synthesised (Scheme 3.6), which was used to make a new hydrazide Ru(II)(bpy)\textsubscript{3} complex 49. This glycine hydrazide Ru(II)(bpy)\textsubscript{3} complex 49 was seemingly more stable to oxygen than the initial Ru(II)(bpy)\textsubscript{3} hydrazide complex 43, and could similarly be used to form hydrazone Ru(II)(bpy)\textsubscript{3} complexes 50. In the mass spectrum less N-N bond cleavage was observed than for the initial Ru(II)(bpy)\textsubscript{3} hydrazone complexes 46, although it was still observable.

Various glycine hydrazone Ru(II)(bpy)\textsubscript{3} complexes 50 could again be formed, however these were less soluble than the hydrazones of the initial hydrazide Ru(II)(bpy)\textsubscript{3} complex scaffold 43. These hydrazone Ru(II)(bpy)\textsubscript{3} complexes 50 were only soluble in DMSO and DMF, while the initial Ru(II)(bpy)\textsubscript{3} complex scaffold hydrazones 46 were soluble in water/acetonitrile mixtures. Upon attempts to dilute DMSO stocks of the new hydrazone Ru(II)(bpy)\textsubscript{3} complexes 50 into water and various buffers in the concentrations required for HRMS analysis, precipitation was observed until 50 \% DMSO was used, even when using more hydrophilic aldehydes, like 4-hydroxy benzaldehyde 45d and 2,5-dihydroxy benzaldehyde 45e in an attempt to aid this solubilisation. Therefore this system also could...
not be used in a biologically relevant setting, so it was decided to look at new potential Ru(II)(bpy)$_3$ complex scaffolds with solubilising groups attached to the core scaffold.

### 3.2.3 New synthetic methodology

As new biologically compatible, stable hydrazone Ru(II)(bpy)$_3$ complex scaffolds were required for DCC, it was decided to see if a new, more divergent synthesis could be exploited.

![Figure 3.3 Difference between linear and divergent synthesis](image)

The syntheses previously described, in Chapter 2, involve the synthesis of separate ligands and protected Ru(II)(bpy)$_3$ complexes for each of the functionalised Ru(II)(bpy)$_3$ complexes required (Figure 3.3a). However, especially for the synthesis of a wide range of Ru(II)(bpy)$_3$ complexes, it would be useful to use a more divergent route, whereby a single Ru(II)(bpy)$_3$ complex precursor could be synthesised and then functionalised (Figure 3.3b).

To this end, an acid functionalised Ru(II)(bpy)$_3$ complex 29 was synthesised (Scheme 3.7),$^{142}$ with the objective of forming amide bonds on its periphery. This acid Ru(II)(bpy)$_3$ complex 29 could be readily synthesised in gram quantities. The carboxylic acid functionality on Ru(II)(bpy)$_3$ complex 29 was hypothesised to be more reactive than that of the diacid ligand 41, due to both an increase in solubility and as the carboxylic acid is more susceptible to attack due to the Ru(II)(bpy)$_3$ core withdrawing electron density. The activation of a carbonyl in this position can be seen in the activation of amide bonds in this position to hydrolysis.
Scheme 3.7 Synthesis of acid functionalised Ru(II)(bpy)$_3$ complex 29

Scheme 3.8 Attempts at amide bond formation on intact Ru(II)(bpy)$_3$ complexes 29 and 30 using peptide coupling agents a) 29 b) 30

Attempts to form amide bonds on the acid Ru(II)(bpy)$_3$ complex 29 using standard peptide coupling agents (Scheme 3.8a), including HATU, HCTU and PyBOP did not prove fruitful, even with week-long reaction times and heating to 60 °C. Similar attempts were also made with a deprotected glycine substituted Ru(II)(bpy)$_3$ complex 30 (Scheme 3.8b), as it was thought that this would be more peptide-like, so that the acid functionality may react more readily with the peptide coupling agents. However, these also did not prove fruitful. These reactions were also difficult to follow: it was found that all the Ru(II)(bpy)$_3$ complexes, except the expected final Ru(II)(bpy)$_3$ complexes 52 and 53 stuck to the baseline of the TLC plate. Similarly LCMS did not prove helpful as the starting (and presumably intermediate) Ru(II)(bpy)$_3$ complexes 29 and 30 did not ionise well, and appeared on the solvent front of the HPLC trace.
Scheme 3.9 Amide bond formation on an intact Ru(II)(bpy)$_3$ complex

Due to the problems encountered using the peptide coupling agents it was decided to attempt to form an acid chloride Ru(II)(bpy)$_3$ complex from the acid Ru(II)(bpy)$_3$ complex 29 and use this to form amide bonds. This route (Scheme 3.9) worked well, giving the fully-functionalised Ru(II)(bpy)$_3$ complexes 41. This reaction proceeded with various different primary amines 38f, i, j and k and even with a secondary amine 38h, and a small aniline 38l, however, reaction with a highly functionalised aniline 38e did not lead to the desired product, presumably due to steric reasons. Ru(II)(bpy)$_3$ complexes formed in this manner also proved to be easier to purify; so long as the amine 38 could be removed by acid washes, only the fully functionalised Ru(II)(bpy)$_3$ complex 41 moved off the baseline on the TLC plate in 10 % methanol in dichloromethane. This is compared to the products from ruthenium(II) complexation from the previous methodology where the Ru(II)(bpy)$_3$ complexes 41 needed to be separated from the ligands 42 which often had similar R$_f$s. This meant that the column purification was much quicker, so removal of acid-sensitive protecting groups as seen with some of the larger Ru(II)(bpy)$_3$ complexes 41c and e on silica was not observed.

There were some disadvantages to this new synthetic route; the yields were much lower than that for ligand formation prior to ruthenium(II) complexation, presumably due to the requirement for 6 amide bond formations on the Ru(II)(bpy)$_3$ complex 29 to form the desired product as opposed to 2 amide bond formations on the bpy ligand 40. However, the advantage of being able to form small quantities of many Ru(II)(bpy)$_3$ complexes much more quickly could allow the acceleration of testing Ru(II)(bpy)$_3$ complexes for a variety of different applications.
Synthetic methods for the synthesis of both 4’ and 5’ monosubstituted Ru(II)(bpy)$_3$ complexes have also been studied, these are presented in Appendix II. The synthesis of these lower functionality Ru(II)(bpy)$_3$ complexes offer the potential advantage of less complicated analytical chemistry in the application of these Ru(II)(bpy)$_3$ complexes for DCC.

### 3.2.4 New Ru(II)(bpy)$_3$ hydrazone scaffolds

![Figure 3.4 Serine (54) and Aspartic/Glutamic acid (55 and 56) hydrazide Ru(II)(bpy)$_3$ complexes, with the hydroxyl/carboxylate groups potentially solubilising the core DCC scaffold](image)

With a new synthetic methodology in hand, it was decided to attempt to improve the solubility of the previously synthesised glycine hydrazone Ru(II)(bpy)$_3$ complexes 50, by increasing the solubility of the core Ru(II)(bpy)$_3$ complex scaffold. It was decided to change the glycine linker in 50 to serine (54), aspartic acid (55) and glutamic acid (56) (Figure 3.4), introducing hydroxyl or carboxylic acid groups to potentially solubilise the whole Ru(II)(bpy)$_3$ complex scaffold.

The addition of the acid groups also had the potential advantage of facilitating the hydrazone exchange by neighbouring group participation. So far nucleophilic catalysts (aniline) for catalysing the hydrazone exchange at neutral pH, have been discussed, however hydrazone exchange can also be catalysed by acid, and indeed ortho-carboxy phenylhydrazine has been shown to increase the rate of hydrazone formation, compared to phenylhydrazine.$^{175}$ In the aspartic acid and glutamic acid hydrazide Ru(II)(bpy)$_3$ complexes 55 and 56 an acid functionality is in close proximity to the hydrazone so this could facilitate the hydrazone exchange, this could be envisaged to work in a manner akin to general acid catalysis in an enzyme active site.
3.2.4.1 Serine- and aspartic acid- hydrazide Ru(II)(bpy)₃ complexes 54 and 55

synthetic attempts

Initially orthogonally protected serine and aspartic acid functionalised Ru(II)(bpy)₃ complexes 41j and k were synthesised (Scheme 3.10), using the new synthetic methodology. From these Ru(II)(bpy)₃ complexes there are two further reactions required to synthesise the desired hydrazide Ru(II)(bpy)₃ complexes 54 and 55, these are:

i) tert-butyl deprotection of the hydroxyl/carboxylic acid side chains and ii) hydrazide formation on the methyl esters. These were attempted in both orders (Scheme 3.11a).

The tert-butyl deprotection proceeded without fault for both the Ru(II)(bpy)₃ complexes 41j and k, however on reaction of these species with hydrazine monohydrate, rather than obtaining the desired hydrazide Ru(II)(bpy)₃ complexes 54 and 55, amide bond cleavage (with 58a) to give the initial hydrazide Ru(II)(bpy)₃ complex 43 or complete degradation (with 58b) were observed. Attempts at first making the hydrazide functionality, yielded less amide bond cleavage on the serine Ru(II)(bpy)₃ complex 41j, however, as there was still observable amide bond cleavage a clean sample of the Ru(II)(bpy)₃ complex 57j could not be obtained. With the aspartic acid Ru(II)(bpy)₃ complex 41k the reaction with hydrazine monohydrate, again yielded degradation. Similar attempts at the hydrazide formation reaction (Scheme 3.11b) on the orthogonally protected serine ligand 59 yielded the highly insoluble hydrazide ligand 48, by amide bond cleavage.
Attempts at hydrazide Ru(II)(bpy)$_3$ complex formation on serine and aspartic acid Ru(II)(bpy)$_3$ complexes 41j and k

3.2.4.2 Using hydrazido-amino acids to functionalise the Ru(II)(bpy)$_3$ complexes

Seemingly the problem with the first attempts at synthesising the serine and aspartic acid hydrazide complexes 54 and 55 was the hydrazide formation reaction. Therefore it was decided to add an amino acid already possessing the hydrazide functionality onto the Ru(II)(bpy)$_3$ complex 29. These amino acids 62 could readily be synthesised from Fmoc-amino acids (Scheme 3.12).

Scheme 3.11 Attempts at hydrazide Ru(II)(bpy)$_3$ complex formation on serine and aspartic acid Ru(II)(bpy)$_3$ complexes 41j and k

Scheme 3.12 Synthesis of serine, aspartic acid and glutamic acid hydrazido-amino acids 62
Scheme 3.13 Attempts at amide bond formation between intact acid functionalised 
Ru(II)(bpy)$_3$ complexes 29, 64 and 65 and the hydrazido amino acids 62 a) disubstituted 
Ru(II)(bpy)$_3$ complex 63, b) monosubstituted Ru(II)(bpy)$_3$ complexes 66 and 67

After synthesising these hydrazido-amino acids 62 it was attempted to add them onto 
the intact acid functionalised Ru(II)(bpy)$_3$ complex core 29 (Scheme 3.13a). Attempts 
were made with all three hydrazido-amino acids 62, but the reactions did not prove 
fruitful, with the aqueous layers staying red, and brown organic phases on aqueous work 
up. Further analysis of the organic phase revealed no discernable mass peaks in the mass 
spectrum, and a brown smudge by TLC, where a single red spot would be expected. Similar 
attempts were made with the 4’ and 5’ monosubstituted Ru(II)(bpy)$_3$ complexes 64 and 
65 (synthesis of these acid functionalised monosubstituted Ru(II)(bpy)$_3$ complexes 64 
and 65 is described in Appendix II) but again this did not yield the desired products 66 
and 67 (Scheme 3.13).
An attempt to add the serine hydrazido-amino acid 62a to the diacid ligand 40 (Scheme 3.14a), did give the desired ligand 68, but with much lower yield than usually observed for similar reactions on the ligand 40. A ruthenium(II) complexation reaction of the ligand 68 was attempted, however the ligand 68 seemingly degraded in the ruthenium(II) complexation reaction.

From this it was thought that perhaps higher temperatures were causing the degradation so the amide bond formation on the intact Ru(II)(bpy)_3 complex 29 was attempted on ice, however this gave similar results to those obtained at room temperature, followed by reflux. A low yield was also observed in attempts to synthesise the 5’ serine hydrazide ligand 70 (Scheme 3.14b).
Scheme 3.14b), with not enough ligand 70 being formed for attempts at ruthenium(II) complexation.

Scheme 3.14 Attempts at synthesising serine hydrazide Ru(II)(bpy)$_3$ scaffolds 63a and 67a by first synthesising ligands 68 and 70, a) disubstituted ligand 68, b) 5’ monosubstituted ligand 70

3.3 Porphyrin scaffolds

Figure 3.5 Porphyrin scaffold a) Tetra-phenyl porphyrin 71  b) Changing from 6 exchangeable groups on the Ru(II)(bpy)$_3$ complexes to 4 on the porphyrin scaffold

Due to the degradation problems experienced in the synthesis of the hydrazide Ru(II)(bpy)$_3$ complexes 54, 55 and 56, a different multivalent hydrazide scaffold was explored. To this end a tetra-phenyl porphyrin scaffold 71 (Figure 3.5a) was chosen. Using this scaffold several advantages can be envisaged: i) there are only 4 exchangeable
groups as opposed to the 6 with the disubstituted Ru(II)(bpy)$_3$ complexes (Figure 3.5b), potentially making the analysis less challenging, ii) the porphyrins primarily ionise as $+1$ rather than $+2$ species separating the peaks in the mass spectrum, and iii) there is no multi-isotopic metal ion, decreasing the isotopic pattern window in the mass spectrum, potentially increasing signal to noise.

3.3.1 Porphyrin scaffold design and synthesis

Attempts were first made to synthesise a scaffold analogous to the initial hydrazide Ru(II)(bpy)$_3$ complex scaffold 43 with the hydrazide directly attached to the tetra-phenyl porphyrin 73 (Scheme 3.15a). This synthesis was attempted via several routes, however determining what happened in the reactions was difficult due to insolubility. Due to these solubility problems and a hypothesised potential for radical degradation of the N-N bond in the mass spectrometer it was decided not to pursue this scaffold any further, and to again incorporate an sp$^3$ centre between the central tetraphenyl porphyrin core and the hydrazide moiety.
Scheme 3.15 Initial attempts at making porphyrin hydrazide scaffolds 73 and 75 a) directly attached to porphyrin 73, b) glycine hydrazide porphyrin 75

At first, a glycine hydrazide porphyrin scaffold 73, analogous to the glycine hydrazide Ru(II)(bpy)₃ complex 49, was chosen. To this end, an ethyl glycine porphyrin 74 was synthesised (Scheme 3.15b), and subjected to hydrazide formation conditions. This gave a very insoluble purple solid, which was hypothesised to be the glycine hydrazide porphyrin 75 but could not be characterised due to its insolubility. The insolubility of the glycine hydrazide porphyrin 75, is perhaps unsurprising given that the deprotected glycine porphyrin is also very insoluble, and hydrazides seem to mirror the solubility of their corresponding acid.
With the solubility issues with the glycine hydrazide porphyrin 75, it was decided to attempt to use a serine in place of the glycine to, as with the Ru(II)(bpy)₃ complexes, increase the scaffold solubility. A serine methyl ester amino acid 62a was first attached to the porphyrin core (Scheme 3.16), however this, as with the Ru(II)(bpy)₃ complexes, did not fair well in the hydrazide formation reaction, therefore it was decided to use the hydrazido-amino acids 62 previously synthesised.
Scheme 3.17 Synthesis of serine, aspartic acid and glutamic acid hydrazide and benzaldehyde hydrazone porphyrins 78 and 79

Amide bonds were formed between the serine, aspartic acid and glutamic acid hydrazido amino acids 62, and tetra-carboxy phenyl porphyrin 72 (Scheme 3.17), using PyBOP as a coupling agent. The hydrazide Boc and side chain tert-butyl protecting groups could readily be removed with TFA to yield the desired hydrazide protecting groups 78. These hydrazide porphyrins were used to form the benzaldehyde hydrazone porphyrins 79. These benzaldehyde hydrazone porphyrins 79 were then used in all further DCC analyses.

3.3.2 Hydrazone exchanges

With the three benzaldehyde hydrazone porphyrins 79 in hand it was first decided to see if it was possible to exchange the benzaldehyde moiety with other aldehydes 44 (Scheme 3.18). The benzaldehyde hydrazone porphyrins 79 were soluble in 10 % DMSO in aqueous solutions, thus allowing attempts at hydrazone exchange to occur in biologically relevant media, in comparison to the attempts with the initial hydrazone Ru(II)(bpy)₃ complexes 46 which required acetonitrile/water mixtures.
As with the Ru(II)(bpy)$_3$ complexes, HRMS was used to follow the hydrazone exchange reactions. The argument for the validity of this method for the Ru(II)(bpy)$_3$ complexes was that as the Ru(II)(bpy)$_3$ complexes are already ionised as a 2+ species, there will be little difference in the ionisation of the different hydrazone Ru(II)(bpy)$_3$ complexes. This argument is not valid for the neutral porphyrins. However, much of the porphyrin ionisation is due to protonation of the central porphyrin core, but protonation of the peripheral groups will now also be more important. This means that the mass spectrum may not give the actual proportions of species in solution, however they will be related. Therefore, if the relative proportions of the peaks in the mass spectrum remain constant, the DCC system has reached a point where the proportions of each species is constant, a static point or a dynamic equilibrium.

Scheme 3.18 Porphyrin hydrazone exchanges a) General reaction scheme, b) Cartoon representation of reaction, c) Cartoon representation of individual exchanges
3.3.2.1 Hydrazine exchange to 1 aldehyde

Figure 3.6 Aldehydes used in hydrazine exchange reactions

Hydrazine exchanges were performed with the three different porphyrin scaffolds 79, with three different aldehydes (Figure 3.6): 2,4-dimethoxy benzaldehyde 44b, 4-carboxy benzaldehyde 44c and 4-methyl ester benzaldehyde 44f. It should be noted that attempts were also made with 4-hydroxy benzaldehyde 44d, however the mass peaks were harder to discern in this case. Hydrazine exchanges were initially performed without a catalyst with the aldehyde added in 50 equivalents compared to the hydrazine porphyrin 79 (12.5 equivalents per hydrazine). These hydrazine exchanges were followed over time (Figure 3.7b), in order to establish i) if hydrazine exchange occurred, ii) if equilibrium was reached and iii) the timescale for the establishment of equilibrium.

Considering one of these systems, (aspartic acid hydrazine porphyrin 79b with 4-carboxy benzaldehyde 44c), hydrazine exchange was observed within 24 hours, and the hydrazine exchanges could be visualised by HRMS (Figure 3.7a), with clearly discerned mass peaks for each of the subsequent hydrazine exchanges being observed. There were some extra peaks in the mass spectrum; these corresponded to sodiated and ammoniated species for each of the subsequent hydrazine exchanges, as well as peaks for N-N bond cleavage. Looking at just the protonated peaks, the progression of these hydrazine exchanges could be followed over time (Figure 3.7b) with successive hydrazine exchanges being observed. The initial species 79b (0) decreased over time, with the species with one hydrazine exchange (1) to 4-carboxy benzaldehyde 44c at first increasing then decreasing. The products of successive hydrazine exchanges (2, 3 and 4) then followed in succession.
Figure 3.7 Exemplary HRMS showing hydrazone exchange on a porphyrin scaffold (79b).
Conditions: 100 μM 79b, 5 mM 44c, 10 % DMSO, 5 mM NH₄OAc, pH 6.75. a) HRMS trace in porphyrin +1 region at start of incubation (top) and after 24 hour incubation (bottom). b) Following the species present over a 24 hour time course.

These hydrazone exchanges were repeated for all three porphyrin hydrazone scaffolds 79 with the three different aldehydes (44b, c and f) (Figure 3.8) with hydrazone exchanges occurring over a 24 hour period. This shows that all 3 porphyrin scaffolds 79 could be compatible for biological DCC, with hydrazone exchange occurring in biologically relevant buffer, at ambient temperature.
Figure 3.8 Initial hydrazone exchange time courses on porphyrin hydrazone scaffolds 79. The separate lines on the graphs correspond to different numbers of benzaldehyde moieties having exchanged for a new aldehyde. 100 μM 79, 5 mM 44, 10 % DMSO in 5 mM NH₄OAc, pH 6.75. a), b) and c) Serine hydrazone porphyrin 79a, d), e) and f) Aspartic acid hydrazone porphyrin 79b, g), h) and i) Glutamic acid hydrazone porphyrin 79c incubated with 4-carboxy benzaldehyde 44c (a), (d) and (g)), 4-methyl ester benzaldehyde 44f(b), (e) and (h)), and 2,4-dimethoxy benzaldehyde 44b ((c), f) and (i)) for 24 hours.

Differing rates of hydrazone exchange were observed between the three porphyrin scaffolds and with the different aldehydes. Generally the glutamic acid and aspartic acid hydrazone porphyrins 79b and c (Figure 3.8d, e, f, g, h and i) showed faster exchange rates than the serine hydrazone porphyrin 79a (Figure 3.8a, b, and c). This supports the hypothesis that the acid functionality may catalyse the hydrazone exchanges. 4-Methyl
ester benzaldehyde 44f and 4-carboxy benzaldehyde 44c also seem to exchange at a faster rate than 2,4-dimethoxy benzaldehyde 44b, this is likely due to the electron-withdrawing nature of the para carbonyl activating the aldehyde/imine to nucleophilic attack, while the electron-donating ortho and para metoxy groups deactivate the aldehyde/imine to attack. The 4-carboxy benzaldehyde 44c also showed faster rates of hydrazone exchange than the 4-methyl ester benzaldehyde 44f.

However in almost all cases equilibration was not being reached within the 24 hour period. Therefore, the addition of a catalyst to the DCC systems to increase the rate of reaching equilibration, was investigated, in the hope to allow equilibration at a timescale more compatible with biological applications.

### 3.3.2.1 Catalysts

As equilibration was not reached within 24 hours, the DCC systems were not completely suitable for protein templation. Different groups have reported the use of nucleophilic catalysts for use in hydrazone exchange reactions in biologically relevant media. Aniline 45 was the initial catalyst reported, and was the catalyst investigated with the Ru(II)(bpy)₃ complexes 46 however, subsequently, different catalysts have been shown to catalyse hydrazone exchanges with faster rates of hydrazone formation and exchange in two different studies.

To look at the effects of catalysis on the hydrazone exchanges, and the rate of reaching equilibrium, aniline 45 and anthranillic acid 80 were chosen, as they are cheap, relatively soluble and are less likely to degrade in DMSO compared to the phenylene diamines reported by Distefano et al. Hydrazone exchange reactions to exchange the benzaldehyde moiety for 4-carboxy benzaldehyde 44c with the three porphyrin scaffolds 79 (Figure 3.9) were carried with both these catalysts.

As can be seen the two catalysts did increase the rate of equilibration in all cases. Comparing the aniline 45 and anthranillic acid 80 catalysts it seems the incubation with aniline 45 may have increased the rate slightly more than that of anthranillic acid 80, therefore aniline 45 was chosen for use in all further studies.

Similar attempts to establish the effects of catalysts on the hydrazone exchanges with 2,4-dimethoxy benzaldehyde 44b and 4-methyl ester benzaldehyde 44f again showed the catalysts increasing the rate of reaching equilibrium. Both catalysts, however, could not be explored with these aldehydes, due to the formation of insoluble imines between the catalysts and the benzaldehyde derivatives. 2,4-Dimethoxy benzaldehyde 44b
precipitated with anthranillic acid 80 and 4-methyl ester benzaldehyde 44f precipitated with aniline 45.

Another important factor when considering catalysis is the catalyst loading. Greaney *et al.* used very high aniline 45 loadings compared to both the aldehydes (2000 eq.) and hydrazides (500 eq. per hydrazone). These catalyst loadings are much higher than can be used in this set up, due to solubility, however different concentrations of catalyst to

Figure 3.9 Effect of catalysis on porphyrin hydrazone exchange, using exemplary reaction with 4-carboxy benzaldehyde 44c on all 3 porphyrin hydrazone scaffolds 79 and addition of no catalyst, aniline 45 and anthranillic acid 80. 100 μM hydrazone porphyrin 79, 5 mM 4-carboxy benzaldehyde 44c, 10 mM catalyst, 10 % DMSO in 5 mM NH₄OAc, pH 6.75 a), b) and c) Serine hydrazone porphyrin 79a, d), e) and f) Aspartic acid hydrazone porphyrin 79b and g), h) and i) Glutamic acid porphyrin 79c, a), d) and g), no catalyst, b), e) and h) aniline 45 catalyst, c), f) and i) anthranillic acid 80 catalyst
aldehyde should have an effect on the rate, especially when the catalyst concentrations are similar to that of the aldehyde. Attempts were made at different aniline 45 loadings, on exchange reactions with 2,4-dimethoxy benzaldehyde 44b, for all three hydrazone porphyrin scaffolds 79, with exemplary results for the aspartic acid hydrazone porphyrin scaffold 79b shown in Figure 3.10. 2,4-dimethoxy benzaldehyde 44b was chosen for this study as it displayed the slowest rates of reaching equilibrium in the initial, no catalyst studies. The rate of reaching equilibrium was faster with increasing catalyst concentrations, as would be expected, however so long as there was catalyst present, equilibrium was reached within 12 hours, a suitable time scale for performing DCC in the presence of a protein. Similar results were obtained for both the serine and glutamic acid hydrazone porphyrins 79a and c, with increasing initial rates being particularly apparent for the serine hydrazone porphyrin 79a. From this serine hydrazone porphyrin 79a data (Figure 3.11) it was seen that under 10 mM aniline 45 concentration hydrazone exchanges occur at a slow rate, with a large rate increase observed at 10 mM aniline, therefore it was decided to use this catalyst concentration for further studies.

Figure 3.10 Effect of catalyst concentration on hydrazone exchanges. Exemplary data with Aspartic acid hydrazone porphyrin scaffold 79b (100 μM) with 2,4-dimethoxy benzaldehyde 44b (5 mM) and varying concentrations of aniline 45 in 10 % DMSO in 5 mM NH₄OAc, pH 6.75
Figure 3.11 Effect of catalyst concentration on serine hydrazone porphyrin scaffold 79a.
Serine hydrazone porphyrin scaffold 79a (100 μM) with 2,4-dimethoxy benzaldehyde 44b (5 mM) and varying concentrations of aniline 45 in 10 % DMSO in 5 mM NH₄OAc, pH 6.75

3.3.2.2 Is this a true dynamic equilibrium or a static mixture?

Having shown that it was possible to exchange the benzaldehyde moiety for another aldehyde, it was necessary to show that the system did not reach a static point and was indeed at a dynamic equilibrium. This was achieved by taking the established mixture from incubation with 25 equivalents of an aldehyde for 24 hours then adding a second 25 equivalents of the same aldehyde to show if it was possible to perturb the equilibrium (Figure 3.12). This was attempted by considering just 2 time points, looking at the distributions following the first 24 hours incubation then 24 hours after the second incubation. This experiment was attempted with 4-carboxy benzaldehyde 44c, using an aniline catalyst for all three hydrazone porphyrin scaffolds 79. If the equilibrium had been perturbed, and the distribution of species changes on second incubation, then the system was indeed in a dynamic equilibrium rather than being static.
Addition of 2 separate batches of aldehyde, incubation with 25 eq. of aldehyde 44c for 24 hours followed by addition of a second 25 eq. of aldehyde 44c. 1st incubation of 100 μM hydrazone porphyrin 79 with 2.5 mM 4-carboxy benzaldehyde 44c and 10 mM aniline 45 in 10 % DMSO in 5 mM NH₄OAc, pH 6.75. 2nd incubation, addition of a further 2.5 mM 4-carboxy benzaldehyde 44c to first incubation. Graphs show an average of 5 separate measurements.

The addition of the second 25 equivalents of aldehyde 44c does indeed change the distribution for all three porphyrin scaffolds 79 (Figure 3.13). With all three hydrazone porphyrin scaffolds 79 there is a shift to more hydrazone exchanges having occurred, with the initial species (0) decreasing and the final species (4) increasing, along with changes in the proportion of intermediary species (1, 2 and 3). As it was possible to perturb the species present, it indicates that the system is at an equilibrium rather than being a static mixture.

3.3.2.3 Hydrazine exchanges to 2 aldehydes

Having shown that a dynamic equilibrium was indeed generated it was decided to incubate the system with 2 different aldehydes, to look at the thermodynamic mixture obtained. Initially this was attempted using the single time point method described above, looking at both mixtures that had been preincubated with one aldehyde followed by
addition of the other aldehyde (Figure 3.14b) as well as ones where a mixture of the two aldehydes was present from the start (Figure 3.14a). For these studies an aniline 45 catalyst with 4-carboxy benzaldehyde 44c and 2,4-dimethoxy benzaldehyde 44b were used with all three hydrazone porphyrin scaffolds 79.

![Figure 3.14 Cartoon depicting 2 methods of performing hydrazone exchange reaction with 2 aldehydes](image)

In all cases the system does give a mixture of different hydrazones (Figure 3.15) as hydrazone exchange occurs with both aldehyde moieties. In the case where one aldehyde has been preincubated with the hydrazone porphyrin 79 followed by addition of the other aldehyde this again showed the establishment of an equilibrium which can be perturbed.

With all three hydrazone porphyrin scaffolds 79, the pre-incubation with 2,4-dimethoxy benzaldehyde 44b followed by addition of 4-carboxy benzaldehyde 44c (Figure 3.15b, e and h) gave a similar distribution of products to that without pre-incubation and direct mixing of the two aldehydes 44b and c from the start (Figure 3.15c, f, and i). This indicates that it is possible to reach the same equilibrium from the two different starting points, indicating these systems are at a thermodynamic equilibrium. There was, however, a bigger difference between the pre-incubation with 4-carboxy benzaldehyde 44c (Figure 3.15a, d and g) and the direct mixing (Figure 3.15c, f and i), this could indicate the system had not yet reached an equilibrium in this set of conditions, and may indicate a longer
time was needed for equilibration to occur. However, some of this discrepancy may come from the method of analysis, for many of these species, especially with incubations with two aldehydes, discerning the signal for the species present above the noise in the mass spectrum could be difficult. This may be leading to the larger discrepancy in this case, as, especially in this system, after the total 48 hour incubation the signal above noise in the
mass spectrum is small. The small signal to noise ratio means that species which have relatively low actual abundance were shown as being present at a much higher percentage than if the signal to noise ratio was much larger.

3.3.2.3.1 *Time to reach equilibrium with 2 aldehydes*

![Figure 3.16](image)

**Figure 3.16** Time for porphyrin hydrazone exchanges to reach equilibrium with two aldehydes, a) Cartoon depicting reaction, b), c) and d) Time course showing the distribution of species with the number of species with each aldehyde functionality over time for the aspartic acid hydrazone porphyrin scaffold 79b after a) Direct mixing of 4-carboxy benzaldehyde and 2,4-dimethoxy benzaldehyde b) Pre-incubation with 4-carboxy benzaldehyde 44c and c) Pre-incubation with 2,4-dimethoxy benzaldehyde 44b. Conditions: 100 μM hydrazone porphyrin 79b, 5 mM each aldehyde 44b and 44c, 10 mM aniline 45, 10 % DMSO, 5 mM NH₄OAc, pH 6.75

Having shown that a mixture of different hydrazone functionalised porphyrins do form on incubation with two aldehydes, the rate of forming this equilibrium was investigated, as in the case of pre-incubation with 4-carboxy benzaldehyde 44c, the rate of reaching the dynamic equilibrium could be different to that with just one aldehyde. This was done by following the hydrazone exchanges with 2 aldehydes over time, with exemplary data for the aspartic acid hydrazone porphyrin 79b shown in Figure 3.16.
In both the pre-incubation and the direct mixing cases an equilibrium was seemingly reached within 12 hours (Figure 3.16). This rate is similar to that with a single aldehyde, and is a reasonable time for protein templation. Again, the species obtained from pre-incubation with 2,4-dimethoxy benzaldehyde 44b and the species obtained from direct mixing of both aldehydes were similar, however the species present after pre-incubation with 4-carboxy benzaldehyde 44c was somewhat different, though the system was seemingly equilibrating. It could be that in this case the species present reach a kinetic trap, with it being difficult to exchange so many 4-carboxy benzaldehydes 44c, or it could again be due to the low signal to noise ratio present in the mass spectrum. However, using the other data obtained, it was possible to see that a dynamic equilibrium is formed from the direct mixing systems, and therefore the system is primed for incubation with a protein.

3.3.3 Protein incubation

![Cartoon depicting incubation with protein. Different distributions of porphyrin hydrazone products are obtained in the presence of a protein compared to its absence.](image)

After showing that hydrazone exchange to generate a dynamic equilibrium on the porphyrin hydrazone scaffolds 79 was possible, incubation with a protein template was attempted. In this case the protein should perturb the equilibrium to enrich compounds which bind to it, as they are now more thermodynamically stable in its presence compared to its absence (Figure 3.17). As a first proof of principle experiment, cytochrome (cyt) c was chosen as the protein. Being a basic protein, it theoretically should enrich porphyrin hydrazones possessing acidic functionality.

Incubation of the three hydrazone porphyrin scaffolds 79 with 2 or 3 additional aldehydes 44 (4-carboxy benzaldehyde, 2,4-dimethoxy benzaldehyde and 3-methyl 2-
carboxaldehyde pyridine) and an aniline 45 catalyst with no protein, with BSA and with cyt c was performed. The incubation with BSA acts as a control to see if the DCL is generating generic protein ligands or more specific cyt c ligands.

Initially a DCL (similar to that in the hydrazone exchange studies) was generated after a 24 hour incubation without the protein template at which point the protein was added and again incubated for 24 hours. After 24 hours the hydrazone exchange was quenched by addition of ammonium hydroxide. Then the separation of protein and the porphyrin DCL was attempted, to allow for analysis. Initially this was attempted using protein concentrators (MWCO 5 kDa) which theoretically should retain the protein, and not the porphyrin DCL. However, the porphyrins stayed in the protein concentrator, even without any protein present. It was then attempted to precipitate the protein from the DCL mixture, using ice-cold ethanol and methanol/chloroform mixtures. These gave some precipitation but mass spectra of the solutions left still showed protein present and did not allow detection of the porphyrin DCL. Analytical HPLC was also attempted on samples with no protein present, however due to the complex mixture of the DCL present the separation of the species requires long separation times, so is not high throughput or compatible with having protein still present in the sample.

3.4 Conclusions

Hydrazone exchange has been shown to be possible on multivalent scaffolds. Initially on a Ru(II)(bpy)$_3$ scaffold, then on a tetraphenyl porphyrin scaffold. Hydrazone exchange, as required for setting up a DCL, was shown to be possible around a Ru(II)(bpy)$_3$ complex hydrazone scaffold 46 in water/acetonitrile mixtures. However, attempts at making this system more biologically compatible did not prove fruitful with solubility and stability/degradation issues. In the process of this study a new synthetic method for 4,4'-disubstituted Ru(II)(bpy)$_3$ complexes was developed, which could prove useful for the development of new Ru(II)(bpy)$_3$ complexes for a variety of different applications.

Subsequently, three porphyrin hydrazone scaffolds 79 have been generated which are more soluble in biologically compatible media (10 % DMSO in aqueous solutions). Hydrazone exchange reactions were performed on these porphyrin scaffolds using several aldehydes, following the reactions over time in order to gain insight into the rates of reaching equilibration. The effects of nucleophilic catalysis using aniline 45 and anthranillic acid 80 have been studied, showing addition of these catalysts lead to equilibration within 12 hours, giving a system capable of reaching equilibrium in a timeframe suitable for templation with biological molecules. The generation of
thermodynamic mixtures after incubation with 2 aldehydes was shown, with equilibration again occurring within 12 hours, giving a system prime for incubation with different protein.

Incubation of all three hydrazone porphyrin scaffolds 79 in a DCL with cyt c was attempted, however separation of the protein and porphyrin DCL proved difficult, and a new method of separating the two will need to be found in order for these incubations to be analysed.
4 Using functionalised Ru(II)(bpy)$_3$ complexes in a protein sensing array

A potential application for functionalised Ru(II)(bpy)$_3$ complexes is as part of an array for the discrimination of different proteins. This works in a similar way to a mammalian nose or tongue, where a relatively small number of receptors can detect a large number of smells, with each smell having a fingerprint-like response often as a result of low affinity binding to many different smell receptors (Figure 4.1), thus allowing discrimination between the different smells. Here the luminescent responses of a range of functionalised Ru(II)(bpy)$_3$ complexes when incubated with different proteins were used to give a fingerprint response for the different proteins, in a 'chemical nose/tongue' approach.

![Figure 4.1](image)

*Figure 4.1* Low affinity binding of an analyte (e.g. smell molecule) to a range of receptors, generates a fingerprint response

4.1 Protein sensing arrays

Sensing arrays have been widely reported for the discrimination of different metal ions, anions and various different small molecules. Arrays for the sensing of large biomolecules, and cells, however, have been less widely reported, but present an interesting target due to the potential for use in multiple applications, including disease diagnosis, and proteome sensing. Traditional approaches for the discrimination of proteins are relatively limited in scope, relying on enzymes and antibody responses, for example using enzyme-linked immunosorbent assays (ELISA), which are cumbersome, expensive and can be plagued with false results, as illustrated by the fact that a study of antibodies for post-translational histone modifications found that 20% of commercially available antibodies failed specificity tests. Therefore it is interesting to see if synthetic molecules can be used as a cheaper, more high throughput and more specific platform for the discrimination of proteins.
In an initial, proof-of-concept experiment, the Hamilton group used functionalized porphyrins to discriminate between cyt c, myoglobin, cyt c551 and ferredoxin to generate fingerprint-like responses for each of the different proteins. They then furthered their study by using a statistical analysis technique, linear discrimination analysis (LDA), to show significant discrimination of cyt c, lysozyme, ferredoxin and α-lactalbumin.

The Anslyn group subsequently described discrimination of proteins and glycoproteins and some ‘within-protein-class’ discrimination of proteins, using combinatorially synthesised tripeptide and boronic acid functionalised hexasubstituted benzenes. They have also used Zn(II)-(dpa) complexes to discriminate between different classes of MAP kinases. The Rotello group have used fluorescent polymers, fluorescent polymer appended gold nanoparticle sensors, and fluorescent protein-appended gold nanoparticle for the detection of a range of proteins and for the detection of metastatic cells. These approaches allowed for quantification of protein concentration and for protein detection within human serum. More recently, array approaches have also been developed for the detection of histone modifications; the Hof group have used different functionalized calixarenes with displaceable indicator dyes to discriminate various different histone post-translational modifications.

### 4.2 Discrimination of proteins using a range of Ru(II)(bpy)₃ complexes

![Figure 4.2 The Ru(II)(bpy)₃ complexes used in the protein sensing arrays](image)

Initially, similar to the Hamilton group studies with functionalised porphyrins, a range of proteins were incubated with a range of Ru(II)(bpy)₃ complexes (Figure 4.2). The Ru(II)(bpy)₃ complexes possess different groups around the periphery and present these groups in different spatial orientations, therefore they should interact with the different protein surfaces in different ways, for example binding with varying affinities, repulsion,
aggregation, disaggregation and affecting the interaction of the Ru(II)\(\text{bpy}\)_3 complex with the well plate (Figure 4.3). All these different interactions can lead to changes in the luminescence intensity of the functionalised Ru(II)\(\text{bpy}\)_3 complexes, with the potential for quenching or enhancement of their luminescence, which can readily be detected.

![Figure 4.3 Cartoon illustrating differential sensing with Ru(II)\(\text{bpy}\)_3 complexes and proteins.](image)

The various combinations of Ru(II)\(\text{bpy}\)_3 complexes and proteins are incubated in a 96 or 384 well plate, with differential interactions between the Ru(II)\(\text{bpy}\)_3 complexes and proteins, for example, different Ru(II)\(\text{bpy}\)_3 complexes binding to the same site or to distal sites on the protein or some Ru(II)\(\text{bpy}\)_3 complexes binding and others not, or even being repelled. These differential interactions lead to differences in the luminescence spectra of the Ru(II)\(\text{bpy}\)_3 complexes.

Eight different Ru(II)\(\text{bpy}\)_3 complexes (26, 29 – 32, 34, 35 and 81) (Figure 4.2) were incubated with nine different proteins (lysozyme, α-chymotrypsin (α-ChT), horse cyt c, papain, ribonuclease (RNase) A, bovine serum albumin (BSA), yeast cyt c, hDM2 and McI-1) (Figure 4.4). The luminescence intensity of each of the Ru(II)\(\text{bpy}\)_3 complexes with each of the proteins was measured and compared to its luminescence intensity without any protein present. The comparison between the presence and absence of protein is
Figure 4.4 X-ray crystal structures of the nine proteins for use in the array, showing the basic (blue) and acidic (red) amino acid residues, molecular weights (MW) and isoelectric points (pI) of the proteins; lysozyme (PDB ID: 1LYZ), α-chymotrypsin (α-ChT) (PDB ID: 4CHA), horse cyt c (1HRC), papain (PDB ID: 9PAP), RNAse A (PDB ID: 5RSA), bovine serum albumin (BSA) (PDB ID: 3V03), yeast cyt c (PDB ID: 2YCC), hDM2 (PDB ID: 4GH7) and Mcl-1 (PDB ID: 5C3F)

Figure 4.5 Differential luminescence responses from the different Ru(II)(bpy)3 complexes 26, 29-32, 34, 35 and 81 (2.5 μM) on incubation with various different proteins (10 μM), (5 mM sodium phosphate, pH 7.5, exc. 467 nm). a) and b) Illustrative luminescence intensity over variable wavelengths for Ru(II)(bpy)3 complex 30 (a) and 26 (b), obtained using plate reader monochromators. c) Fingerprint changes in luminescence responses from all the different Ru(II)(bpy)3 complexes with the different proteins, illustrated as percentage differences from incubation with and without protein, responses measured using plate reader fixed filters (exc. 467 nm, em. 625 nm)
necessary as the magnitude of the intensities of the Ru(II)(bpy)$_3$ complexes varies between plates.

For each of the proteins a fingerprint-like response (Figure 4.5) with different luminescence responses from each of the Ru(II)(bpy)$_3$ complexes on addition of different proteins was obtained. Most of the Ru(II)(bpy)$_3$ complexes show varying luminescence quenching on incubation, and hence interaction with, the proteins. Ru(II)(bpy)$_3$ complex 34 shows varying degrees of enhanced luminescence intensity on interaction with each of the different proteins. This indicates that this may be a method in which it is possible to discriminate between different proteins.

4.2.1 Statistical analysis

From the bar chart in Figure 4.5c, it can be seen that there are differences in luminescence response to the individual proteins with the different Ru(II)(bpy)$_3$ complexes, however the discrimination of the proteins, by eye, is not trivial, especially if the number of Ru(II)(bpy)$_3$ complexes or proteins were to be increased. Therefore it is necessary to simplify the data from 8 dimensional data (arising from the 8 Ru(II)(bpy)$_3$ complexes) to a more manageable number of dimensions, 2 or 3 dimensions which can readily be readily visualised on a scatter graph.

**Figure 4.6** PCA/LDA aims to take the 8 dimensional Ru(II)(bpy)$_3$ complex array data down to two (or three) dimensions by taking linear combinations of the original data, thus allowing the data to be plotted on a 2-D (or 3-D) scatter graph, with the aim of getting clusters for the different analytes (proteins)

Two different statistical techniques, principal component analysis (PCA) and discriminant analysis (DA), have been widely used in the literature for reducing the dimensionality of array data. These two techniques generate score plots using combinations of the original data on each of the axes, in order to take the n-dimensional
data down to 2 or 3 dimensions, allowing the data to be plotted graphically (Figure 4.6).\textsuperscript{188} Both techniques reduce the data using matrix techniques, finding eigenvectors and eigenvalues to describe new axes and the level of discrimination respectively. Matrix derivations and discussion of the mathematics behind the techniques are discussed in more detail in Appendix III.

DA is a supervised statistical method,\textsuperscript{189} meaning it uses a training set of data, and looks for the best way of organising the data so as to increase the discrimination between classes while decreasing the variation within classes. PCA is unsupervised and looks at finding the maximum variation between all the data, irrespective of the data classes.\textsuperscript{190} This means that DA looks for clustering of classes whereas PCA spreads data points out as much as possible.\textsuperscript{188} As the ultimate aim with this analysis is to see if it is possible to cluster all the data from each protein, and separate that cluster from that of the other proteins, discriminant analysis has been used.

In this case linear discriminant analysis (LDA) was used, taking linear combinations of the original components as described earlier. This makes an assumption that all the covariance matrices for all the different classes are equivalent, i.e. how much the data for a single protein/Ru(II)(bpy)\textsubscript{3} complex combination compares with another protein/Ru(II)(bpy)\textsubscript{3} complex combination within the same replicate is the same across different replicates for the same protein. This means that it is assumed that the luminescence responses for a specific Ru(II)(bpy)\textsubscript{3} complex with a protein should be the same across replicates. Quadratic discriminant analysis (QDA) does not make this assumption, finding quadratic surfaces rather than linear combinations of the original data.\textsuperscript{188} However, QDA requires many more replicates to be performed as it requires more parameters to be derived from the data set.\textsuperscript{191}

4.2.1.1 **Linear discriminant analysis on Ru(II)(bpy)\textsubscript{3} complex array with a panel of proteins**

Initially a 2-D LDA was performed (Figure 4.7a) on the array data obtained. This showed clear and distinct clusters for the two cyt c proteins (horse heart and yeast cyt c). This is a promising result, as cyt c is a highly evolutionarily conserved protein so separation of cyt c from two different species could be seen as difficult to achieve. Clustering was also seen for the other proteins, but these clusters are all located within the same region. The use of 3-D LDA was then explored, in the hope of discriminating between these clusters. The 3-D LDA (Figure 4.7b) again shows distinct clusters for the cyt c proteins, and clustering of the other proteins. However, the clustering of the other
proteins is again in one region, with little discrimination between them, therefore array was changed in order to increase the discrimination between these proteins.

![Figure 4.7](image)

**Figure 4.7** LDA for the Ru(II)(bpy)$_3$ complex-protein array, a) 2-D analysis, b) 2 views of the 3-D analysis

### 4.3 Using 2 fluorophores to enhance protein discrimination

As all the proteins could not be discriminated using just the Ru(II)(bpy)$_3$ complexes the use of a second fluorophore to produce better discrimination was investigated. With two different fluorophores present a much more complicated network of interactions becomes possible leading to changes in the luminescence of the two separate fluorophores (Figure 4.8).

Array studies with molecules possessing multiple fluorophores have been reported by the Margulies group, where they synthesised complicated molecules in order to detect a variety of different analytes, including a range of pharmaceuticals in urine samples,\(^{192}\) and more recently different aggregation states of β-amyloid peptides.\(^{193}\) The molecules used in the Margulies group studies are large and require considerable synthetic effort, however it was thought that it may be possible to achieve the same goal of using multiple interacting
Figure 4.8 Array of potential different interactions between the two fluorophores and different proteins, including interactions between the two luminescent molecules, binding on different sites on the protein, masking of binding sites for one molecule by the other molecule, docking of the two molecules together on the protein.

fluorophores by using different luminescent molecules already synthesised within the Wilson group. To this end the Ru(II)(bpy)₃ complexes described previously and a FITC-NOXA B peptide (sequence FITC-(Ga)AAQLARIGDKVNLRLKLN-NH₂), which had been synthesised by Dr. Katherine Horner, were used. Fluorescein is a logical fluorophore choice in this case; although its maximum excitation wavelength 494 nm, is higher than that used for the Ru(II)(bpy)₃ complexes (467 nm), 467 nm is still within its excitation spectrum and can therefore be used, allowing simultaneous visualisation of the luminescence of both the Ru(II)(bpy)₃ complexes (emission 625 nm) and the fluorescent peptide (emission 520 nm).

4.3.1 Deciding on appropriate concentrations

Due to the differences in quantum yield of the Ru(II)(bpy)₃ complexes and the fluorescein moiety, appropriate concentrations of the species to be ascertained, which i)
allowed the luminescence of both species to be visualised, ii) didn’t have overlap of the emission bands for the two fluorophores, iii) didn’t require high concentrations of protein and iv) would be responsive to protein recognition.

Figure 4.9 Concentration test to determine appropriate concentration of FITC-NOXA B peptide to use with 2.5 μM Ru(II)(bpy)$_3$ complex. Luminescence emission intensity at variable wavelengths (exc. 467 nm) upon incubation of 2.5 μM Ru(II)(bpy)$_3$ complex 29 with various concentrations of FITC-NOXA B peptide in 5 mM sodium phosphate, pH 7.5

2.5 μM Ru(II)(bpy)$_3$ complex solution and 10 μM protein were the concentrations selected for further study as these concentrations worked well with the Ru(II)(bpy)$_3$ array, showing specific responses to different proteins, even if they could not be discriminated. Therefore it was only necessary to decide on a compatible concentration of the FITC-NOXA B peptide. The fluorescence of various concentrations of the FITC-peptide with 2.5 μM of Ru(II)(bpy)$_3$ complexes 29 was characterised (Figure 4.9). From this, it was decided to use 0.5 μM FITC-NOXA B in future array studies as it had similar luminescence intensity to the Ru(II)(bpy)$_3$ complexes at 2.5 μM, with excitation at 467 nm, and allowed the maxima for both emission bands to be visualised.

4.3.2 Discrimination of proteins

After deciding on the concentrations to use, an array with 6 different proteins (cyt c, BSA, RNase A, papain, α-ChT and lysozyme) was performed, and the luminescence of the Ru(II)(bpy)$_3$ complexes and the FITC-NOXA B peptide recorded and compared to that without the protein present. The incubation with the different proteins, again gave rise to differential responses from the Ru(II)(bpy)$_3$ complexes (Figure 4.10), as well as the FITC-
NOXA B peptides, giving a fingerprint-like response for the different proteins (Figure 4.11).

Figure 4.10 Luminescence response (exc. 467 nm, in 5 mM sodium phosphate, pH 7.5, 2 hour incubation) of the FITC-NOXA B peptide alone (a), Ru(II)(bpy)$_3$ complex 26 alone (b), FITC-NOXA B with Ru(II)(bpy)$_3$ complex 26 (c) and FITC NOXA B with Ru(II)(bpy)$_3$ complex 30 (d), showing differences in the spectra.

Generally the luminescence intensities of the different Ru(II)(bpy)$_3$ complexes was quenched (by variable amounts) on addition of the various different proteins, as in the Ru(II)(bpy)$_3$ complex-protein array discussed earlier. In the majority of cases the fluorescence intensity of the FITC-NOXA B peptide was enhanced. This is as would be expected for any binding to a protein surface as fluorescein is a solvatochromic fluorophore, changing its fluorescence dependent on the solvent (or local environment). Differences in its spectral properties dependent on polarity,$^{194}$ the state of hydrogen bonding in its local environment,$^{195}$ and pH.$^{196}$ Both hydrogen bonding and the polarity will be affected by binding to a protein surface.
Figure 4.11  Fingerprint luminescent responses for the 6 different proteins on incubation of Ru(II)(bpy)$_3$ complexes alone (a) and (b)), Ru(II)(bpy)$_3$ complexes with FITC-NOXA B peptide (c), (d), (e) and (f)), looking at both the fluorescein (emission 520 nm) (e) and (f)) and Ru(II)(bpy)$_3$ channels (emission 630 nm) (c), (d), (e) and (f)) on excitation at 467 nm where appropriate. In 5 mM sodium phosphate, pH 7.5 buffer, 2.5 μM Ru(II)(bpy)$_3$ complex, 0.5 μM FITC-NOXA B peptide and 10 μM protein where appropriate, readings using fixed filters. a), c) and e) 2 hour incubation, b), d) and f) 20 hour incubation.

Another factor which was identified as important for this array was incubation time. Data was collected after 2 hour and 20 hour incubations (Figure 4.11a, c and e and b, d and f respectively) of the same plate, with subtly different responses obtained after these two different incubations. The data obtained after both these incubations was
reproducible, indicating that after both these incubations, the system was indeed at equilibrium, however there was a different equilibrium present at these times. These differences might be expected for the two proteases used in the arrays (α-ChT and papain), as these can potentially degrade both themselves and the FITC-NOXA B peptide at different rates, however differences are observable for all the proteins, this shows that the system is more complicated. For example, it could be that incubation for long periods of time with the Ru(II)(bpy)$_3$ complexes and the FITC-NOXA B leads to changes in protein structure, indeed it has been shown that the Ru(II)(bpy)$_3$ complex 26 destabilises cyt c, lowering its melting temperature.$^{144}$

### 4.3.2.1 LDA of 6 protein, 2 fluorophore array

![Figure 4.12](image.png)

**Figure 4.12** 2-D LDA for the two fluorophore array with 6 proteins, a) 2 hour incubation data, b) 20 hour incubation data, c) Combined 2 and 20 hour incubation data

The data from these arrays were analysed using LDA. In two dimensions (Figure 4.12), looking at the 2 discriminants which define the most between class variation, it is seen that all the proteins are within distinct clusters, for the 2 hour incubation, 20 hour incubation and the combined data. In all three cases the data for the different proteins cluster together, with the cyt c always being in a distinct separate cluster. After 2 hours the BSA cluster is separate from the other protein clusters, and after 20 hours the RNAse A
cluster is separate from the other proteins. Combining the 2 hour and 20 hour incubation data shows separation of both of these proteins, however there is still significant overlap between α-ChT and lysozyme and papain and lysozyme. To separate these protein clusters further 3-D LDA was used (Figure 4.13), assigning the 3rd linear discriminant (F3) as the z axis.

Figure 4.13 3-D LDA of 2 fluorophore array data and Ru(II)(bpy)$_3$ complex array for 6 proteins (combined 2 hour and 24 hour incubation data). a) 2 fluorophore array showing separation of the clusters for the 6 proteins, b) LDA of Ru(II)(bpy)$_3$ complex array with the same 6 proteins, doesn’t show separation of the clusters for the same 6 proteins.

The 3-D LDA (Figure 4.13a), again, shows the clearly defined clusters and now shows separation of the clusters for all the proteins, thus showing that using this technique it is possible to discriminate these 6 proteins. Compared to the original Ru(II)(bpy)$_3$ array (Figure 4.13b), where there is only discrimination of cyt c from the other proteins, there is a clear and distinct difference with the addition of this second fluorophore, allowing for much better discrimination of the different proteins. Looking at some subsets of the data obtained for the two fluorophore array, for example only considering the 2 hour or the 20 hour incubation readings or only considering the Ru(II)(bpy)$_3$ or FITC bands, do not allow this level of discrimination, showing a requirement to use all the data obtained.
It is possible to plot confidence ellipsoids (Figure 4.14), to see the confidence of discrimination between the different proteins. For this 6 protein array it is seen that at the 95 % confidence level (Figure 4.14a) the ellipsoids are clearly distinct but have some overlap, this means that at the 95 % confidence level the proteins are not completely distinguished from each other. However, at the 80 % confidence level (Figure 4.14b), the ellipsoids are distinct from each other, showing significant discrimination between the proteins at this level.

Figure 4.14 Confidence ellipsoids for the LDA of the 6 protein, 2 fluorophore array a) 95 % confidence ellipsoids, b) 80 % confidence ellipsoids

4.3.3 Including more therapeutically interesting proteins

Having had success in discriminating commercially available proteins, more therapeutically interesting proteins were added to the array, namely Mcl-1 and hDM2, which had been prepared by Kirstin Spence, so as to demonstrate the diagnostic potential of the approach. The protein incubations were performed in the same manner as for the original array.

A 3-D LDA was performed with the data obtained for all the proteins, again showing clearly defined clusters of each of the proteins, however there was considerable overlap of the clusters for some of the proteins, with all the data for 5 of the proteins (BSA, α-ChT, papain, RNase A and lysozyme) existing on a straight line parallel to the z (F3) axis. Using other discriminants (the fourth and fifth discriminants) to replace the first, second or both discriminants, still did not separate these clusters.
Figure 4.15 3-D LDA of 2 fluorophore array data after addition of Mcl-1 and hDM2 incubations

From all the LDA performed, cyt c was always well separated from all the other proteins; this could mean that the separation of cyt c from all the other data dominates in the LDA, therefore it was hypothesised that it might be possible to separate the other proteins if cyt c was removed from the analysis. Upon removing the cyt c data from the analysis (Figure 4.16) it was observed that the clusters were much more effectively separated, demonstrating that it may be possible to discriminate the different proteins.

Figure 4.16 3-D LDA of 2 fluorophore array after removal of the cyt c data from the analysis

Looking at the 80 % and 95 % confidence ellipsoids (Figure 4.17) for these data shows ellipsoids for Mcl-1 and hDM2 distinct from the other proteins, but overlapping each other. The ellipsoids for both of these proteins are quite large as many fewer replicates were performed with these data compared to with the other proteins (7 replicates for Mcl-1 and 9 for hDM2, compared to ~30 for the other proteins). More replicates for both of these proteins may distinguish these two proteins more readily. The other protein confidence ellipsoids are much smaller but show some overlap at both the 80 % and 95 % confidence levels, showing that while the clusters are distinct from each other, complete discrimination of the proteins is not achieved. However the small amounts of overlap
between the different protein ellipsoids mean that if the luminescence data were to be obtained for an unidentified protein, it would likely be attributed to the correct protein.

![Figure 4.17](image)

**Figure 4.17** Confidence ellipsoids for the LDA of 2 fluorophore-protein array, excluding the cyt c data a) 95% confidence ellipsoids, b) 80% confidence ellipsoids

### 4.4 Conclusions

Attempts were made to discriminate various different proteins using the luminescence responses of 8 different Ru(II)(bpy)$_3$ complexes on incubation with the proteins, giving a fingerprint-like response for the different proteins. However, on performing a LDA on the data, yeast cyt c and horse heart cyt c could readily be discriminated from each other and the other proteins, but all the other proteins clustered within the same region, and could not be discriminated from each other, even in 3-D. To increase the potential for discrimination the arrays were incubated with a second fluorophore, a FITC-NOXA B peptide. This generated a more complicated fingerprint response and allowed for the discrimination of 6 different commercially available proteins, when using 3-D LDA. The addition of the more therapeutically interesting proteins, Mcl-1 and hDM2 decreased this discrimination, however if the cyt c data, which seemed to dominate the LDA separation in all cases, was removed some discrimination could again be achieved. Therefore, a platform has been generated for the discrimination of different proteins using two fluorophores,
which does not necessarily require the lengthy synthesis of molecules akin to those used by the Margulies group.¹⁹³,¹⁹⁷–¹⁹⁹
5 Thesis summary and future work

This thesis has focussed on the development of multivalent scaffolds (namely Ru(II)(bpy)$_3$ complexes and tetraphenyl-porphyrins) for their use as protein surface mimetics. The focus has centred on understanding how they interact with proteins, designing new platforms for the development of high affinity ligands and using them for new applications in protein detection and discrimination.

In Chapter 2, the utility of the Ru(II)(bpy)$_3$ scaffold to effectively mimic a native protein-protein interaction (PPI) was established. One Ru(II)(bpy)$_3$ complex was shown to effectively mimic the cytochrome (cyt) c/cyt c peroxidase PPI, binding to cyt c with the same binding site, similar thermodynamic and electrostatic binding profile as cyt c peroxidase. A larger, more functionalised Ru(II)(bpy)$_3$ complex was shown to enhance the interactions of this native PPI, with increased electrostatic interactions with cyt c, leading to an increase in the enthalpic contribution to binding. This showed how it is possible to enhance the interactions of a native PPI using synthetic molecules, in order to generate high affinity ligands for protein surfaces. Using this knowledge the rational design of new protein surface mimetics, based on both the Ru(II)(bpy)$_3$ scaffold and other multivalent scaffolds for binding to new, more therapeutically interesting protein targets could be established. The new synthetic methodology for the synthesis of these Ru(II)(bpy)$_3$ complexes, presented in Chapter 3, could aid in this endeavour, allowing for a much more expedient synthesis of a wide range of Ru(II)(bpy)$_3$ complexes for testing against different proteins.

Hydrazone-based dynamic combinatorial chemistry around three porphyrin scaffolds was presented in Chapter 3, showing that it is possible to generate a dynamic equilibrium in biologically relevant media. This system is prime for incubation with protein and for the generation of high affinity protein ligands. A proof-of-principle incubation with cyt c did not prove fruitful as the protein could not be separated from the (quenched) porphyrin dynamic combinatorial library, therefore a method for separation of the porphyrin DCL and protein needs to be established. This will firstly show if it is indeed possible to generate ligands for the model protein cyt c, and then allow the incubation with other, harder to target, proteins to see if it is possible to generate high affinity ligands for other protein surfaces.

In Chapter 4, an array approach for the discrimination of different proteins by incubation with two fluorophores (Ru(II)(bpy)$_3$ complexes and a FITC-labelled NOXA B
peptide) was presented. This allowed the discrimination of a range of different commercially available proteins, and showed some discrimination with the more therapeutically interesting proteins, Mcl-1 and hDM2. This array could be expanded to include additional therapeutically interesting proteins, to see if it is possible to discriminate a range of proteins of therapeutic interest. The use of further FITC labelled peptides, or the addition of a third fluorophore may aid in this, and lead to a tool for quick discrimination and later identification of a wide range of proteins.

Another potential platform for the discrimination of different proteins is to combine the ideas presented in Chapter 3 and 4, using the composition of a dynamic combinatorial library generated in the presence of different proteins, to discriminate between the proteins. Initial experiments in this vain are presented in Appendix IV, using a hydrazide functionalised Ru(II)(bpy)$_3$ complex in the presence of a range of aldehydes to discriminate between three different proteins. This approach requires more repeats to establish if it is a method capable of discriminating the different proteins. Subsequently the discrimination of a wider range of proteins will need to be established, and potential investigations into the use of different dynamic combinatorial chemistry scaffolds for this application, for example the porphyrin scaffolds presented in Chapter 3.

An additional avenue for future study with the Ru(II)(bpy)$_3$ complexes is to use them as PPI stabilisers. Initial results (presented in Appendix V) suggest that two of the synthesised Ru(II)(bpy)$_3$ complexes can stabilise the quaternary structure of a mutant variant (R337H) of the p53 tetramerisation domain. Further work on this showing that the p53 tetramerisation domain is indeed stabilised, for example by proteolysis experiments and showing binding affinity and binding site will show if these Ru(II)(bpy)$_3$ complexes can be used as PPI stabilisers as well as PPI inhibitors.
6 Experimental section

6.1 Synthesis

6.1.1 General considerations

Reagents and solvents were purchased from major suppliers and used without further purification. Anhydrous chloroform, dichloromethane, and methanol were obtained from the in-house solvent purification system, from Innovative Technology Inc. PureSolv®, other solvents used were of HPLC grade. Water for aqueous solutions was deionised.

Thin layer (silica) chromatography was performed using Merck Kiesegel 60 F254 0.25 mm precoated aluminium plates. Product spots were visualised by colour and under UV light (254 nm and 365 nm). Flash column chromatography was performed using silica gel 60 (0.043 – 0.063 mm VWR or Sigma Aldrich) or alumina (Brockman I from Sigma Aldrich), unless otherwise stated silica gel was used and pressure was applied by means of head bellows.

1H NMR spectra were obtained on Bruker DPX 300 (300 MHz), Avance 500 (500 MHz) or DRX500 (500 MHz) spectrometers and referenced to either residual non-deuterated solvent peaks or tetramethylsilane. 13C spectra were recorded on a Bruker DPX 300 (75 MHz) Bruker or an Avance 500 (126 MHz) and referenced to the solvent peak. 1H spectra are reported as follows: 1H NMR (spectrometer frequency, solvent) δ ppm to 2 d.p. (multiplicity, J coupling constant in Hertz, number of protons, assignments). Chemical shifts (δ) are quoted in ppm with signal splitting recorded as singlet (s), doublet (d), triplet (t), quartet (q), quintet (qu.) multiplet (m) and broad (br.). Coupling constants (J) are measured to the nearest 0.1 Hz. Similarly, 13C spectra are reported as follows: δ (spectrometer frequency, solvent) δ ppm to one decimal place. Assignments of spectra were assisted by the results of DEPT, COSY, HMQC and HMBC experiments. 13C NMR spectra were obtained for all novel, and most literature, small molecules and ligands. Where possible 13C spectra were obtained for porphyrin molecules, however due to the large molecular size and thus broadness of peaks in the 1H NMR, 13C NMR spectra were not able to be obtained for the Ru(II)(bpy)3 complexes and some porphyrins. For the larger protected Ru(II)(bpy)3 complexes it was found necessary to use DMSO-d6 for discernment of peaks in the 1H NMR spectra, despite solubility in CDCl3 and MeOD.
Infrared spectra were recorded on a Perkin Elmer Fourier-Transfer spectrometer. Spectra were analysed neat and structurally important absorptions are quoted. Absorption maxima (ν max) are quoted in wavenumbers (cm⁻¹).

HPLC LC-MS were recorded on a Bruker HCT ultra under electrospray ionisation (ESI) conditions. High resolution mass spectra were recorded on a Bruker Daltonics microTOF Premier Mass Spectrometer, under positive ESI conditions unless otherwise stated.

6.1.2 Synthetic protocols

2,2’-Bipyridine-4,4’-dicarboxylic acid, 40¹⁴²,²⁰⁰,²⁰¹

Potassium dichromate (9.10 g, 30.9 mmol) was added to a stirred solution of 4,4’-dimethyl-2,2’-bipyridine, 39 (2.00 g, 10.9 mmol) in concentrated sulfuric acid (50 mL) at 70°C over 2 hours, keeping the temperature between 70 and 80 °C. After the addition, the hot solution was poured onto ice (200 g) and the ice allowed to melt. The off-white precipitate formed was isolated by vacuum filtration. The solid was redissolved in 50 % nitric acid (50 mL) and the solution heated under reflux for 2 hours. The resulting mixture was cooled and poured onto ice (200 g) and the white precipitate isolated by vacuum filtration to yield the product as a white solid (2.23 g, 9.13 mmol, 84 %); ¹H NMR (300 MHz, TFA-d₆) δ ppm 8.36 (d, J = 5.5 Hz, 2 H, H₃), 8.93 (d, J = 5.5 Hz, 2 H, H₂), 9.03 (s, 2 H, H₁), 11.42 (s, 2 H, COO-H); IR (solid state, cm⁻¹) 2435, 1707; ESI-MS m/z found 245.0558 [M+H]+, [C₁₂H₉N₂O₄]+ requires 245.0557

4,4’-Dimethylester-2,2’-bipyridine, 47¹⁴²

Thionyl chloride (20 mL) was added dropwise to a stirred solution of 2,2’-bipyridine-4,4’-dicarboxylic acid, 40 (3.00 g, 12.3 mmol) in anhydrous methanol (200 mL) under a nitrogen atmosphere and the resulting solution heated under reflux for 20 hours. The reaction was then quenched with saturated sodium hydrogen carbonate solution (100
mL), and the mixture extracted with chloroform (3 × 100 mL). The combined organic phases were dried (sodium sulfate), and the solvent removed to yield the crude product as a pale orange solid, this was recrystallized in chloroform to yield the product as pale pink crystals (2.10 g, 7.72 mmol, 63 %); ¹H NMR (300 MHz, CDCl₃) δ ppm 4.02 (s, 6 H, H4), 7.93 (d, J = 4.9 Hz, 2 H, H3), 8.89 (d, J = 4.9 Hz, 2 H, H2), 8.98 (s, 2 H, H1); ¹³C NMR (126 MHz, CDCl₃) δ ppm 52.8, 120.6, 123.3, 138.6, 150.2, 156.5, 165.6; IR (solid state, cm⁻¹) 1726 (C=O ester); ESI-MS m/z found 273.0870 [M+H]⁺, [C₁₄H₁₃N₂O₄]⁺ requires 273.0870

Ru(II)(DMSO)₄Cl₂₁⁵₆

Ru(III)Cl₃.xH₂O (1.00 g) in anhydrous dimethylsulfoxide (5 mL) was degassed for 30 minutes. The solution was then heated under reflux under a nitrogen atmosphere for 30 minutes. Half of the solvent was then boiled off and the mixture cooled to room temperature. The reaction mixture was then diluted with acetone (10 mL) and the yellow precipitate isolated. To yield the product as a yellow solid (1.89 g, 3.91 mmol); ESI-MS m/z found 501.9317 [M+NH₄]⁺, [C₈H₂₈NO₄S₄Ru]⁺ requires 501.9322. (Poor solubility of this compound in all but DMSO meant NMR spectra could not be obtained)

Tris (4,4’-dimethylester-2,2’-bipyridine) ruthenium(II) dihexafluorophosphate, ⁴⁹₁⁴₂

4,4’-Dimethylester-2,2’-bipyridine, ⁴⁰ (382 mg, 1.40 mmol), Ru(II)(DMSO)₄Cl₂ (150 mg, 0.310 mmol), silver nitrate (105 mg, 0.620 mmol) and ethanol (20 mL) were heated under reflux for 7 days. The red solution was filtered and the filtrate concentrated. The red solid was redissolved in water (15 mL) and an excess of ammonium hexafluorophosphate added. The resultant red precipitate was isolated (203 mg, 0.168 mmol, 54 %); ¹H NMR (300 MHz, Acetone) δ ppm 2.88 (s, 18 H, H4), 7.98 (d, J = 5.9 Hz, 6 H, H2), 8.40 (d, J = 5.9 Hz, 6 H, H3), 9.38 (s, 6 H, H1); IR (solid state, cm⁻¹) 1726 (C=O ester); ESI-MS m/z found 459.0720 [M]²⁺, [C₄₂H₃₆N₆O₁₂Ru]²⁺ requires 459.0717

105
Tris \( (2,2'\text{-bipyridine-4,4'}\text{-dicarboxylic acid}) \) ruthenium(II) dichloride, \( 29^{142} \)

\[
\text{Ru(II)}\left(\begin{array}{c}
\text{H} \\
\text{O} \\
\text{N} \\
\text{O} \\
\text{C} \\
\text{H}
\end{array}\right)2\text{Cl}
\]

\( \text{Tris}\ (4,4'\text{-dimethylester-2,2'-bipyridine})\) ruthenium(II) dihexafluorophosphate, \( 49 \) (1.00 g, 0.828 mmol) in ethanol (20 mL) and 1 M sodium hydroxide solution (20 mL) was stirred for 1 hour. The solution was neutralized with 1 M hydrochloric acid and concentrated. The salt was removed by dialysis (MWCO 0.1 - 0.5 kDa) against pure water to yield the product as a red solid (702 mg, 0.776 mmol, 94 %); \( ^1\text{H} \text{NMR (300 MHz, D}_{2}\text{O}) \delta \text{ ppm} 7.52 \ (d, J = 5.7 \text{ Hz}, 6 \ H, \text{H2}), \ 7.73 \ (d, J = 5.7 \text{ Hz}, 6 \ H, \text{H3}), \ 8.67 - 8.78 \ (\text{br. s,} \ 6 \ H, \text{H1}); \ IR \ (\text{solid state, cm}^{-1}) \ 3305 \ (\text{O-H}), \ 1600 \ (\text{C=O acid}); \ ESI-MS \ m/z \text{ found} \ 417.0246 \ [\text{M}^{2+}; \ [\text{C}_{36}\text{H}_{34}\text{N}_{6}\text{O}_{12}\text{Ru}]^{2+} \text{ requires} \ 417.0248 \)

**Ethyl 2-[(2-{4- [(2-ethoxy-2-oxoethyl) carbamoyl] pyridin-2-yl} pyridin-4-yl) formamido] acetate, 41f**

\[
\text{HN}
\]

\( 2,2'\text{-Bipyridine-4,4'}\text{-carboxylic acid, 40 \ (1.50 g, 6.14 mmol) and thionyl chloride (20 mL) were heated under reflux for 16 hours, the solvent was then removed in vacuo, and the dry acid chloride flushed with nitrogen and used immediately. To the dry acid chloride was added anhydrous chloroform (40 mL), ethyl glycine hydrochloride salt (1.89 g, 13.5 mmol) and triethylamine (1.88 mL, 13.5 mmol) and the reaction mixture heated under reflux, under a nitrogen atmosphere, for 16 hours. The reaction was then cooled and concentrated to yield the crude product as a pink solid, which was purified by flash column chromatography (5 % methanol in dichloromethane) to yield the product as a beige solid (1.52 g, 3.65 mmol, 60 %); \( ^1\text{H} \text{NMR (500 MHz, CDCl}_3) \delta \text{ ppm} \ 1.37 \ (t, J = 7.2 \text{ Hz}, 4 \ H, \text{H7}), \ 4.24 - 4.40 \ (m, 8 \ H, \text{H5 and H6}), \ 7.93 \ (d, J = 4.8 \text{ Hz}, 2 \ H, \text{H2}), \ 8.82 \ (s, 2 \ H, \text{H1}), \ 8.91 \ (d, J = 4.8 \text{ Hz}, 2 \ H, \text{H3}); \ ^{13}\text{C} \text{NMR (126 MHz, DMSO-\text{d}_6) \delta ppm} \ 14.1, 41.4, 60.6, 118.2, 121.9, 106 \)
142.1, 150.2, 155.6, 165.1, 169.5; IR (solid state, cm\(^{-1}\)) 3303 (N-H), 1741 (C=O ester), 1648 (C=O amide); ESI-MS \(m/z\) found 415.16203 [M+H]\(^+\), [C\(_{20}\)H\(_{23}\)N\(_4\)O\(_6\)]\(^+\) requires 415.1612

*Tris* (ethyl 2-\{2-\{4-\{2-ethoxy-2-oxoethyl\}carbamoyl\}pyridin-2-yl\}pyridin-4-yl)formamidoacetate) ruthenium(II) diX, 42f

**Method 1**

Ethyl 2-\{2-\{4-\{2-ethoxy-2-oxoethyl\}carbamoyl\}pyridin-2-yl\}pyridin-4-yl)formamidoacetate, 41f (410 mg, 0.991 mmol), Ru(II)(DMSO)\(_2\)Cl\(_2\) (150 mg, 0.310 mmol) and silver nitrate (105 mg, 0.620 mmol) in ethanol (20 mL) were heated under reflux for 7 days. The resulting solution was cooled to room temperature, filtered through celite, the and the celite pad washed thoroughly with dichloromethane until no more red compound could be seen. The red filtrate concentrated to yield the crude product as a red solid. This was purified by flash column chromatography (5 % methanol in dichloromethane) to yield the dinitrate salt as a red solid (426 mg, 0.290 mmol, 94 %).

**Method 2**

*Tris* (2,2'-bipyridine-4,4'-dicarboxylic acid) ruthenium(II) dichloride, 29 (50 mg, 0.055 mmol) in thionyl chloride (10 mL) and dimethylformamide (1 drop) was heated under reflux for 18 hours. The reaction mixture was then concentrated \textit{in vacuo} and flushed with nitrogen to yield the acid chloride as a red solid. The acid chloride was resuspended in anhydrous chloroform (30 mL) and ethyl glycine hydrochloride salt (94 mg, 0.50 mmol) and diisopropylethylamine (0.17 mL, 0.99 mmol) were added. The reaction mixture was heated under reflux for 18 hours. The reaction mixture was then cooled to room temperature and washed with saturated sodium hydrogen carbonate solution (30 mL), 1 M hydrochloric acid (30 mL) and brine (30 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a red solid. This was purified by flash column chromatography (10 % methanol in dichloromethane) to yield the dichloride salt as a red solid (56 mg, 0.039 mmol, 72 %).
\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 1.16 (t, \(J = 6.8\) Hz, 18 H, H7), 3.97 - 4.18 (m, 48 H, H5 + H6), 7.71 (br. s, 6 H, H2), 9.12 (s, 6 H, H1), 9.34 (br. s, 6 H, H3); IR (solid state, cm\(^{-1}\) 3256, (N-H), 1734 (C=O ester), 1664 (C=O amide); ESI-MS m/z found 672.1840 [M]\(^2+\), [C\(_{60}\)H\(_{66}\)N\(_{12}\)O\(_{18}\)Ru]\(^2+\) requires 672.1834

**Tris** (2-{[2-[(carboxymethyl) carbamoyl] pyridin-2-yl] pyridin-4-yl}formamido)acetic acid) ruthenium(II) dichloride, 30

Tris (ethyl 2- {[2-4-[(2-ethoxy-2-oxoethyl) carbamoyl] pyridin-2-yl] pyridin-4-yl}formamido)acetate) ruthenium(II) dinitrate, 42f (200 mg, 0.136 mmol) was dissolved in ethanol (5 mL), and water (5 mL), 1 M sodium hydroxide solution (5 mL) was added, and the resulting mixture stirred for 18 hours. The reaction mixture was then neutralized with 1 M hydrochloric acid and concentrated to yield the product as a red solid in a mixture with sodium chloride. This mixture was redissolved in a minimum amount of water and dialysed (MWCO 0.1 – 0.5 kDa) against pure water to yield the product as a dark red solid (173 mg, 0.133 mmol, 98 %); \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) ppm 3.95 (s, 12 H, H5), 7.73 (dd, \(J = 5.9, 1.6\) Hz, 6 H, H2), 7.94 (d, \(J = 5.9\) Hz, 6 H, H1), 8.99 (app. s, 6 H, H3); IR (solid state, cm\(^{-1}\) 3223 (N-H), 3251 (O-H), 1644 (C=O acid), 1585 (C=O amide); ESI-MS m/z found 588.0885 [M]\(^2+\), [C\(_{40}\)H\(_{42}\)N\(_{12}\)O\(_{18}\)Ru]\(^2+\) requires 588.0819

(2S,2'R)-tetra-tert-butyl-2,2'-{([2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl)} disuccinate, 41g
2,2’-Bipyridine-4,4’-dicarboxylic acid, 40 (100 mg, 0.400 mmol), triethylamine (1 drop) and thionyl chloride (10 mL) were heated under reflux for 16 hours. The mixture was cooled to room temperature and the solvent removed in vacuo to yield the acid chloride as an orange-red solid. The dry acid chloride was then redissolved in anhydrous chloroform (20 mL) and added dropwise to a stirred solution of di-tert butyl L-aspartic acid hydrogen chloride salt (253 mg, 0.901 mmol) and triethylamine (0.25 mL, 1.80 mmol) in anhydrous chloroform at 0 °C, under a nitrogen atmosphere. The reaction mixture was warmed to room temperature and heated under reflux for 48 hours. The mixture was cooled to room temperature and the solvent removed to yield the crude product as a brown oil. This was purified by flash column chromatography (3 % - 6 % methanol in chloroform) to yield the product as a yellow solid (262 mg, 0.375 mmol, 91 %); ¹H NMR (300 MHz, CDCl₃) δ ppm 1.49 (s, 18 H, H7/H8), 1.52 (s, 18 H, H7/H8), 2.91 (dd, J = 17.2, 4.3 Hz, 2 H, H6), 3.04 (m, J = 17.2, 4.3 Hz, 2H, H6’), 4.92 (dt, J = 7.5, 4.3 Hz, 2 H, H5), 7.46 (d, J = 7.5 Hz, 2 H, H4), 7.77 (dd, J = 5.0, 1.7 Hz, 2 H, H2), 8.78 (app. s, 2 H, H1), 8.83 (d, J = 5.0 Hz, 2 H, H3); ¹³C NMR (126 MHz, CDCl₃) δ ppm 28.0, 28.1, 37.5, 49.7, 81.9, 82.8, 118.0, 121.8, 142.3, 150.1, 156.3, 165.1, 169.5, 170.2; IR (solid state, cm⁻¹) 3346 (N-H), 1723 (C=O ester), 1650 (C=O amide); ESI-HRMS: found m/z 699.3615 [M+H]⁺, [C₃₆H₅₁N₄O₁₀]⁺ requires 699.3599

**Tris ((2S,2’R)- tetra- tert-butyl- 2,2’- ([[2,2’-bipyridine]- 4,4’- dicarboxylicyl) bis (azanediyl)) disuccinate) ruthenium(II) dichloride, 42g**

(2S,2’R)-tetra-tert-butyl-2,2’-(([[2,2’-bipyridine]-4,4’-dicarboxyl]bis[azanediyl]) disuccinate, 41g (300 mg, 0.429 mmol), Ru(II)(DMSO)₄Cl₂ (65 mg, 0.134 mmol), silver nitrate (46 mg, 0.268 mmol) and ethanol (20 mL) were heated under reflux for 7 days. After which time the reaction mixture was filtered hot and concentrated. The red solid was then dissolved in a minimum amount of ethanol and loaded onto an SP Sephadex column and eluted with 1:1 acetone: 0.1 M sodium chloride solution and all the red fractions collected and concentrated. The combined red fractions were redissolved in acetone and filtered to remove sodium chloride, and this was repeated until no more white salt was visible in the concentrated sample. The Ru(II)(bpy)₃ complex was then purified by flash
chromatography (1 – 3 % methanol in chloroform) and the red fractions collected. These were concentrated, redissolved in chloroform and extracted into water, until the organic phase was no longer red. The combined aqueous phases were concentrated to yield the product as a red solid (77 mg, 0.034 mmol, 25 %); \(^1\)H NMR (300 MHz, Acetone) \(\delta\) ppm 2.10 (app. s, 108 H, H7 + H8), 2.81 - 3.07 (m, 12 H, H6), 4.79 - 5.07 (m, 6 H, H5), 7.89 (br. m, 6 H, H3), 8.37 (br. m, 6 H, H2), 8.82 - 8.98 (br. m, 6 H, H1); IR (solid state, cm\(^{-1}\)) 3310 (N-H), 1725 (C=O ester), 1710 (C=O ester), 1673 (C=O amide); ESI-HRMS: found \(m/z\) 1098.4854 [M]\(^{2+}\), \([C_{108}H_{150}N_{12}O_{30}Ru]\(^{2+}\) requires 1098.4822; \(\lambda_{\text{max}}\) (MeOH): 306 nM (\(\varepsilon\) \(\text{dm}^3\) mol\(^{-1}\) cm\(^{-1}\) 240 723 981)

**Tris** (2S,2'R)-2,2'-([(2,2'-bipyridine]-4,4'-dicarbonyl)bis(azanediyl))disuccinic acid) ruthenium(II) ditrifluoroacetate, 31

![Chemical Structure](image)

**Tris** ((2S,2'R)-tetra-tert-butyl-2,2'-([(2,2'-bipyridine]-4,4'-dicarbonyl)bis(azane-diyl)] disuccinate) ruthenium(II) dichloride, 42g (68 mg, 0.030 mmol), trifluoroacetic acid (4.5 mL) and water (0.5 mL) were stirred for 3 days. The reaction mixture was then concentrated in vacuo to yield the product as a red-black solid (57 mg, 0.029 mmol, 98 %); \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) ppm 2.67 (br. s, 6 H, H6), 2.78 (br. s, 6 H, H6'), 4.61 (br. s, 6 H, H5), 7.70 (br. s, 6 H, H3), 7.90 (br. s, 6 H, H2), 8.97 (br. s, 6 H, H1); IR (solid state, cm\(^{-1}\)) 3181 (O-H), 1648 (C=O acid, amide); ESI-HRMS: found \(m/z\) 762.1081 [M]\(^{2+}\), \([C_{60}H_{54}N_{12}O_{30}Ru]\(^{2+}\) requires 762.1056

(2S,2'S)-tetra-tert-butyl 2,2'-(5-nitroisophthaloyl)bis(azanediyli)disuccinate, 37b\(^{143}\)

![Chemical Structure](image)

5-nitroisophthalic acid (1.00 g, 4.74 mmol) and dimethylformamide (1 drop) in thionyl chloride (5 mL) were heated under reflux for 4 hours. The reaction mixture was
concentrated *in vacuo* to yield the acid chloride as a white solid. The acid chloride, under a nitrogen atmosphere, was redissolved in anhydrous dichloromethane (50 mL) and ditert-butyl L-aspartic acid. Hydrogen chloride salt (2.94 g, 10.4 mmol) and triethylamine (1.45 mL, 10.4 mmol) were added. The reaction mixture was stirred at room temperature, under a nitrogen atmosphere, for 16 hours. The resulting solution was then washed with 1 M hydrochloric acid (50 mL), saturated sodium hydrogen carbonate solution (50 mL) and brine (50 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a beige solid, this was purified by flash column chromatography (3:7 ethyl acetate:dichloromethane) to yield the product as a white solid (1.50 g, 2.25 mmol, 48 %); 

\[ ^1H \text{NMR (300 MHz, CDCl}_3 \] δ ppm 1.38 (s, 18 H, H6/H7), 1.42 (s, 18 H, H6/H7), 2.78 (dd, \( J = 17.2, 4.3 \text{ Hz, } 2 \text{ H, H4} \)), 2.94 (dd, \( J = 17.2, 3.9 \text{ Hz, } 2 \text{ H, H4} ' \)), 4.70 - 4.92 (m, 2 H, H5), 7.35 (m, 2 H, H3), 8.52 (app. s, 1 H, H1), 8.70 (d, \( J = 1.5 \text{ Hz, } 2 \text{ H, H2} \)); 

\[ ^13C \text{NMR (126 MHz, CDCl}_3 \] δ ppm 27.9, 28.1, 37.3, 50.0, 82.0, 83.0, 124.7, 131.4, 136.4, 148.5, 163.8, 169.4, 170.3; IR (solid state, \( \text{cm}^{-1} \) ) 3371 (N-H), 1734 (C=O ester), 1656 (C=O amide);

**ESI-MS m/z found 688.3052 \([M+Na]^+\), \([C_{32}H_{50}N_3O_{12}Na]^+\) requires 688.5052**

\( (2S,2'S)\)-tetra-tert-butyl 2,2’-((5-aminoisophthaloyl)bis(azanediyl))disuccinate, 38b

(2S,2 ’S)-tetra-tert-butyl 2,2’-((5-nitroisophthaloyl)bis(azanediyl))disuccinate, 37b

(1.00 g, 1.65 mmol) was dissolved in methanol (20 mL) and ethyl acetate (20 mL). The reaction mixture was degassed and palladium on activated charcoal (11 mg) was added and the solution degassed again. The solution was then put under a hydrogen atmosphere and stirred for 16 hours. The solution was then filtered twice and concentrated to yield the product as a flocculent cream solid (981 mg, 1.54 mmol, 94 %); 

\[ ^1H \text{NMR (500 MHz, CDCl}_3 \] δ ppm 1.48 (s, 18 H, H6/H7), 1.53 (s, 18 H, H6/H7), 2.88 (dd, \( J = 17.0, 4.4 \text{ Hz, } 1 \text{ H, H5} \)), 2.99 (dd, \( J = 17.0, 4.5 \text{ Hz, } 1 \text{ H, H5} ' \)), 4.83 - 4.99 (m, 1 H, H4), 7.33 (m, 2 H, H3), 7.54 (s, 2 H, H2), 7.77 (s, 1 H, H1); 

\[ ^13C \text{NMR (126 MHz, CDCl}_3 \] δ ppm 27.9, 28.0, 37.5, 49.7, 81.6, 82.4, 114.9, 116.6, 135.5, 147.3, 166.6, 169.9, 170.3; IR (solid state, \( \text{cm}^{-1} \) ) 3341 (N-H), 1725 (C=O ester), 1671 (C=O ester), 1648 (C=O amide); ESI-MS m/z found 636.3500 \([M+H]^+\), \([C_{32}H_{50}N_3O_{10}]^+\) requires 636.3491
1,4-Di-tert-butyl (2S)-2-[[3-[[2S]-1,4-bis(tert-butoxy)-1,4-dioxobutan-2-yl] carbamoyl] -5- [2- (4- {[3,5-bis ([[(2S)-1,4-bis (tert-butoxy)-1,4- dioxobutan-2-yl] carbamoyl]) phenyl] carbamoyl} pyridin-2-yl) pyridine-4-amido] phenyl) formamido]butanedioate, 41b

2,2'-Bipyridine-4,4'-dicarboxylic acid, 40 (128 mg, 0.524 mmol) was heated under reflux in thionyl chloride (10 mL) for 18 hours, and the solvent removed in vacuo. The resultant acid chloride was flushed with nitrogen and used immediately. The acid chloride was redissolved in anhydrous chloroform (15 mL) and heated to reflux, under a nitrogen atmosphere. A solution of (2S,2'S)-tetra-tert-butyl 2,2'-(5-aminoisophthaloyl) bis(azanediyl)) disuccinate (600 mg, 0.943 mmol), 38b and anhydrous triethylamine (0.16 mL, 1.15 mmol) in anhydrous chloroform (35 mL), under a nitrogen atmosphere, was added dropwise to the refluxing solution and the resulting mixture heated under reflux for 18 hours. The solvent was then removed to yield the crude product as a pink solid, which was purified by flash column chromatography (1 – 5 % methanol in dichloromethane) to yield the product as a beige solid (110 mg, 0.0743 mmol, 14 %); 1H NMR (500 MHz, DMSO-\textit{d}6) \( \delta \) ppm 1.42 (s, 36 H, H10/H11), 1.44 (s, 36 H, H10/H11), 2.72 (dd, \( J = 16.2, 7.3 \) Hz, 4 H, H9), 2.85 (dd, \( J = 16.2, 7.3 \) Hz, 4 H, H9'), 4.77 (q, \( J = 7.3 \) Hz, 4 H, H8), 8.05 (d, \( J = 6.5 \) Hz, 2 H, H3), 8.08 (s, 2 H, H6) 8.44 (s, 4 H, H5), 8.90 (d, \( J = 6.5 \) Hz, 2 H, H2), 9.01 (s, 2 H, H1), 11.07 (app. s, 2 H, H4); 13C NMR (126 MHz, DMSO-\textit{d}6) \( \delta \) ppm 27.5, 27.7, 37.0, 78.7, 81.1, 104.5, 118.6, 121.9, 122.4, 122.6, 135.0, 138.7, 142.9, 150.3, 155.5, 164.1, 165.8, 169.2, 169.7; IR (solid state, cm\(^{-1}\)) 3343 (N-H), 1726 (C=O ester), 1671 (C=O ester), 1656 (C=O amide); ESI-MS \textit{m}/\textit{z} found 1479.7185 [M+H]+, [C\textsubscript{76}H\textsubscript{100}N\textsubscript{8}O\textsubscript{22}]\(^+\) requires 1479.7181
Tris (1,4-di-tert-butyl(2S)-2-[(3-[[[(2S)-1,4-bis(tert-butoxy)-1,4-dioxobutan-2-yl] carbamoyl]-5-[2-{(4-[[[(2S)-1,4-bis(tert-butoxy)-1,4-dioxobutan-2-yl] carbamoyl]} phenyl] carbamoyl] pyridin-2-yl) pyridine-4-amido]phenyl) formamido] butanedioate) ruthenium(II) dinitrate, 42b

1,4-Di-tert- butyl (2S)-2- [(3- [[[2S]- 1,4- bis (tert- butoxy)- 1,4- dioxobutan-2-yl] carbamoyl]-5-[2-{(4-[[[(2S)-1,4-bis(tert-butoxy)-1,4-dioxobutan-2-yl] carbamoyl]} phenyl] carbamoyl] pyridin-2-yl)pyridine-4-amido]phenyl)formamido]butanedioate, 41b (400 mg, 0.270 mmol), Ru(II)(DMSO)Cl₂ (42 mg, 0.0872 mmol), and silver nitrate (92 mg, 0.540 mmol) in ethanol (30 mL) were heated under reflux for 6 days. The solution was then filtered through celite and the filtrate concentrated. The resulting red solid was purified by flash column chromatography (5 % methanol in dichloromethane) to yield the product as a red solid (50 mg, 0.0107 mmol, 12 %); ¹H NMR (500 MHz, DMSO-d₆) δ ppm 1.39 (br. s, 108 H, H10/H11), 1.40 - 1.52 (br. s, 108 H, H10/H11), 2.63 - 2.76 (br. m, 12 H, H9), 2.77 - 2.92 (br. m, 12 H, H9'), 4.76 (q, J = 7.7 Hz, 12 H, H8), 8.03 - 8.15 (m, 24 H, H7 + H2 + H3), 8.35 - 8.53 (m, 18 H, H5, H6), 8.76 - 8.94 (m, 12 H, H1 + H4); IR (solid state, cm⁻¹) 3274 (N-H), 1729 (C=O ester), 1666 (C=O amide); ESI-MS m/z found 2270.5083 [M]²⁺, [C₂₂₈H₃₀₅N₂₀₆O₆Ru]²⁺ requires 2269.0185

1,4-Dimethyl (2S)-2-[(3-[[[(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl] carbamoyl]-5-nitrophenyl)formamido]butanedioate, 37a¹42
5-nitroisophthalic acid (2.00 g, 9.47 mmol) and dimethylformamide (1 drop) in thionyl chloride (20 mL) were heated under reflux for 18 hours. The solvent was removed in vacuo to yield the acid chloride as a white solid. The acid chloride was redissolved in anhydrous dichloromethane (40 mL), dimethyl L-aspartic acid hydrochloride salt (4.12 g, 20.8 mmol) and triethylamine (2.92 mL, 20.8 mmol) were added and the reaction mixture stirred for 16 hours. The resulting solution was quenched with saturated sodium hydrogen carbonate solution (50 mL). The organic phase was then washed with 1 M hydrochloric acid (50 mL) and brine (50 mL), dried (sodium sulfate) and concentrated to yield the crude product as a beige solid, this was purified by flash column chromatography (3:7 ethyl acetate:dichloromethane) to yield the product as a white solid (2.54 g, 5.11 mmol, 54%); \(\text{\textsuperscript{1}H NMR (500 MHz, CDCl}_3\text{)} \delta \text{ppm 3.03 (dd, } J = 17.4, 6.1 \text{ Hz, } 2 \text{H, H5), 3.18 (dd, } J = 17.4, 4.8 \text{ Hz, 2 H, H5'), 3.74 (s, 6 H, H6/H7), 3.84 (s, 6 H, H6/H7), 5.06 - 5.18 (m, 2 H, H4), 7.65 (d, } J = 7.9 \text{ Hz, 3 H, H3), 8.60 (t, } J = 1.5 \text{ Hz, 1 H, H1), 8.79 (d, } J = 1.5 \text{ Hz, 2 H, H2); }\)

13C NMR (126 MHz, CDCl_3) δ ppm 35.9, 49.4, 52.3, 53.1, 125.2, 131.4, 135.8, 148.5, 164.0, 171.0, 171.5; IR (solid state, cm\(^{-1}\)) 3386 (N-H), 1747 (C=O ester), 1727 (C=O amide); ESI-MS m/z found 498.1368 [M+H]+, [C\(_{20}\)H\(_{24}\)N\(_3\)O\(_{12}\)]+ requires 498.1354

1, 4- dimethyl (2S)-2- [[3- amino-5-{{(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl}carbamoyl}phenyl]formamido]butanedioate, 38a

1,4-dimethyl (2S)-2-[[3-{{(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl} carbamoyl}-5-nitrophenyl]formamido]butanedioate, 37a (1.00 g, 2.14 mmol) in methanol (30 mL) and ethyl acetate (30 mL) was degassed and palladium on activated charcoal (20 mg) added. The solution was degassed again and put under a hydrogen atmosphere. The mixture was stirred for 18 hours, then filtered twice and concentrated to yield the product as a cream solid (1.07 g, 2.29 mmol, quant); \(\text{\textsuperscript{1}H NMR (500 MHz, CDCl}_3\text{)} \delta \text{ppm 3.00 (dd, } J = 17.4, 4.9 \text{ Hz, 2 H, H5), 3.10 (dd, } J = 17.4, 6.5 \text{ Hz, 2 H, H5'), 3.71 (s, 6 H, H6/H7), 3.80 (s, 6 H, H6/H7), 5.06 (dt, } J = 6.5, 4.9 \text{ Hz, 2 H, H4), 7.18 (s, 2 H, H2), 7.45 (s, 1 H, H1), 7.46 - 7.48 (m, 2 H, NH\(_2\)); }\)

13C NMR (126 MHz, CDCl_3) δ ppm 36.1, 49.1, 52.2, 52.9, 115.0, 116.6, 135.1, 147.4, 166.7, 171.3, 171.6; IR (solid state, cm\(^{-1}\)) 3360 (N-H), 1747 (C=O ester), 1643 (C=O amide); ESI-MS m/z found 468.1624 [M+H]+, [C\(_{20}\)H\(_{26}\)N\(_3\)O\(_{10}\)]+ requires 468.1613
1,4-Dimethyl (2S)-2-([3-[2-[(4-{[3,5- bis ([(2S)-1,4- dimethoxy-1,4- dioxobutan-2-yl] carbamoyl]) phenyl] carbamoyl} pyridin-2-yl]pyridine-4-amido]-5-[(2S)-1,4- dimethoxy-1,4-dioxobutan-2-yl]carbamoyl]phenyl)formamido)butanedioate 41

2,2′-Bipyridine-4,4′-dicarboxylic acid, 40 (471 mg, 1.93 mmol) in thionyl chloride (15 mL) was heated under reflux for 18 hours. The solvent was then removed in vacuo, and the resulting acid chloride was redissolved in anhydrous chloroform (10 mL) and heated under reflux, under a nitrogen atmosphere. A solution of 1,4-dimethyl (2S)-2-[(3-amino-5-[(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl]carbamoyl]phenyl)formamido]butanedioate, 38a (1.62 g, 3.47 mmol) and anhydrous triethylamine (0.59 mL, 4.3 mmol) in anhydrous chloroform (20 mL), kept under a nitrogen atmosphere was added dropwise to the refluxing solution and the solution then heated under reflux for 16 hours. The reaction mixture was concentrated and the resulting pink crude product purified by flash column chromatography (5 % methanol in chloroform) to yield the product as a beige solid (1.41 g, 1.23 mmol, 64 %); $^1$H NMR (500 MHz, DMSO-$d_6$) δ ppm 2.90 (dd, $J = 16.4, 8.0$ Hz, 4 H, H9), 3.01 (dd, $J = 16.4, 8.0$ Hz, 4 H, H9), 3.64 (s, 12 H, H10/H11), 3.67 (s, 12 H, H10/H11), 4.91 (app. q, $J = 8.0$ Hz, 4 H, H8), 8.06 (dd, $J = 5.1, 1.3$ Hz, 2 H, H2), 8.12 (app. s, 2 H, H6), 8.46 (d, $J = 1.3$ Hz, 4 H, H5), 8.98 – 9.04 (m, 4 H, H1 + H3), 9.15 (d, $J = 7.6$ Hz, 4 H, H7), 11.09 (s, 2 H, H4); $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ ppm 35.3, 49.4, 51.7, 52.3, 118.6, 122.1, 122.4, 122.8, 134.6, 138.8, 142.9, 150.3, 155.6, 164.1, 165.8, 170.5, 171.1; IR (solid state, cm$^{-1}$) 3306 (N-H), 3011 (N-H), 1731 (C=O ester), 1651 (C=O amide), 1598 (C=O amide); ESI-MS m/z found 572.1751 [M+H]$^{2+}$, [C$_{52}$H$_{56}$N$_8$O$_{22}$]$^{2+}$ requires 572.1749
Tris (1,4-dimethyl (2S)-2-{{3-[2-{4-[[3,5-bis{[[2S]-1,4-dimethoxy-1,4-dioxobutan-2-yl]carbamoyl}] phenyl] carbamoyl} pyridin-2-yl] pyridine-4-amido]-5- {{(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl} carbamoyl} phenyl} formamido) butanedioate) ruthenium(II) dinitrate, 42b

1,4-Dimethyl (2S)-2- {{3-[2-{4-[[3,5-bis{[[2S]-1,4-dimethoxy-1,4-dioxobutan-2-yl]carbamoyl}] phenyl] carbamoyl} pyridin-2-yl] pyridine-4-amido]-5- {{(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl} carbamoyl} phenyl} formamido) butanedioate, 41a (1.00 g, 0.875 mmol), Ru(II)(DMSO)$_4$Cl$_2$ (132 mg, 0.273 mmol) and silver nitrate (93 mg, 0.547 mmol) in ethanol (20 mL) were heated under reflux for 7 days. The reaction mixture was then filtered and the red filtrate concentrated to yield the crude product as a red solid. This was purified by flash column chromatography (5 % - 10 % ethanol in dichloromethane) to yield the product as a red solid (625 mg, 0.171 mmol, 63 %); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ ppm 2.89 (dd, $J = 16.3, 6.7$ Hz, 12 H, H9), 2.99 (dd, $J = 16.3, 6.7$ Hz, 12 H, H9'), 3.43 (br. s, 12 H, H7), 3.64 (s, 36 H, H10/H11), 3.67 (s, 36 H, H10/H11), 4.89 (q, $J = 6.7$ Hz, 7 H, H8), 8.06 - 8.16 (m, 6 H, H6), 8.18 (d, $J = 6.5$ Hz, 6 H, H2) 8.45 (s, 12 H, H5), 9.15 (d, $J = 6.5$ Hz, 6 H, H3), 9.58 (br. s, 6 H, H1), 11.23 (app. s, 6 H, H4); IR (solid state, cm$^{-1}$) 3293 (N-H), 2953 (N-H), 1731 (C=O ester), 1656 (C=O amide), 1599 (C=O amide); ESI-MS $m/z$ found 1764.9596 [M+H]$^{2+}$, [C$_{156}$H$_{162}$N$_2$O$_{66}$Ru]$^{2+}$ requires 1764.4551
Tris (2S)-2-{{3-[2-(4-[[3,5-bis([[(1S)-1,2-dicarboxyethyl] carbamoyl]} phenyl] carbamoyl]pyridin-2-yl]pyridine-4-amido]-5-[[1(1S)-1,2-dicarboxyethyl]carbamoyl]phenyl]formamido)butanedioic acid) ruthenium(II) dichloride, 26

Tris (1,4-dimethyl (2S)-2-{{3-[2-(4-[[3,5-bis([[2S)-1,4-dimethoxy-1,4-dioxobutan-2-yldicarboxyethyl]carbamoyl}])phenyl]carbamoyl]pyridin-2-yl]pyridine-4-amido]-5-[[1(2S)-1,4-dimethoxy-1,4-dioxobutan-2-yl]carbamoyl]phenyl]formamido)butanedioate) ruthenium(II) dinitrate, 42b (15 mg, 0.0041 mmol), lithium hydroxide (5 mg, 0.021 mmol), tetrahydrofuran (2 mL) and water (2 mL) were stirred for 1 hour. The solution was then neutralized by addition of 1 M hydrochloric acid. The red solution was concentrated, and dialysed (MWCO 0.1 – 0.5 kDa) against pure water, to yield the product as a red solid (11 mg, 0.0032 mmol, 82 %); \( ^1 \)H NMR (500 MHz, D\(_2\)O) \( \delta \) ppm 2.68 - 2.78 (m, 24 H, H\(_9\)), 4.71 (m, 12 H, H\(_8\)), 7.94 (d, \( J = 5.4 \) Hz, 6 H, H\(_2\)), 7.99 - 8.04 (br. s, 6 H, H\(_6\)), 8.09 (br. d, \( J = 5.4 \) Hz, 6 H, H3), 8.11 - 8.21 (br. s, 12 H, H5), 8.93 - 9.31 (br. s, 6 H, H1); IR (solid state, cm\(^{-1}\)) 3255 (N-H), 2549 (O-H acid), 1625 (C=O acid), 1601 (C=O amide); ESI-HRMS found \( m/z \) 1596.211 [M]\(^{2+}\), 1596.2672 [C\(_{132}\)H\(_{114}\)N\(_{24}\)O\(_{66}\)Ru]\(^{2+}\) requires 1596.2672

Ethyl 2-{{3-amino-5-[[bis (2-ethoxy-2-oxoethyl) carbamoyl]phenyl]-N-(2-ethoxy-2-oxoethyl)formamido}acetate, 38c

Ethyl 2-{{3-[bis (2-ethoxy-2-oxoethyl) carbamoyl]-5-nitrophenyl]-N-(2-ethoxy-2-oxoethyl)formamido}acetate, 37b (synthesised by Georgina Pleasance, 974 mg, 1.76 mmol) was dissolved in methanol (20 mL) and ethyl acetate (20 mL) and the solution

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degassed. Palladium on activated charcoal (20 mg) was added and the solution degassed again. The solution was put under a hydrogen atmosphere and stirred for 18 hours. The reaction mixture was then filtered twice and concentrated to yield the product as a sticky pale yellow solid (823 mg, 1.57 mmol, 89%); 1H NMR (500 MHz, CDCl₃) δ ppm 1.23 (t, J = 7.1 Hz, 9 H, H5), 1.28 (t, J = 7.1 Hz, 9 H, H5'), 4.07 (s, 8 H, H3), 4.13 (q, J = 7.1 Hz, 4 H, H4), 4.22 (q, J = 7.1 Hz, 4 H, H4'), 6.75 (s, 1 H, H1), 6.79 (s, 2 H, H2); 13C NMR (126 MHz, CDCl₃) δ ppm 14.1, 14.2, 47.4, 51.6, 61.4, 61.7, 113.8, 114.5, 136.4, 147.5, 168.8, 169.0, 171.5; IR (solid state, cm⁻¹) 3361 (N-H), 1734 (C=O ester), 1642 (C=O amide); ESI-MS m/z found 524.2214 [M+H]⁺, [C₂₄H₃₄N₃O₁₀]⁺ requires 524.2239

2-(1-{3-[Bis(2-ethoxy-2-oxoethyl)carbamoyl]-5-[2-[4-{[3,5-bis[bis(2-ethoxy-2-oxoethyl)carbamoyl]phenyl]carbamoyl]pyridin-2-yl]pyridine-4-amido}phenyl}-N-(2-ethoxy-2-oxoethyl)formamido)-1-(ethylperoxy)ethylidene, 41c

2,2'-Bipyridine-4,4'-dicarboxylic acid, 40 (181 mg, 0.743 mmol) in thionyl chloride (10 mL) was heated under reflux for 18 hours. The solvent was then removed in vacuo and the yellow acid chloride flushed with nitrogen and used immediately. The acid chloride was redissolved in anhydrous chloroform (10 mL) and heated to reflux under a nitrogen atmosphere. A solution of ethyl 2-(1-{3-amino-5-[bis(2-ethoxy-2-oxoethyl)carbamoyl]phenyl}-N- (2-ethoxy-2-oxoethyl) formamido)acetate, 38c (702 mg, 1.34 mmol) and anhydrous triethylamine (0.23 mL, 1.63 mmol) in anhydrous chloroform (15 mL) was added dropwise to the refluxing solution and the resulting solution heated under reflux for 24 hours. The reaction mixture was then concentrated to yield the product as a pale pink solid (1.05 g, 0.836 mmol, quant.). 1H NMR (500 MHz, DMSO-d₆) δ ppm 1.20 (t, J = 7.2 Hz, 12 H, H9), 1.22 (t, J = 7.0 Hz, 12 H, H9'), 4.08 (s, 8 H, H7), 4.14 (q, J = 7.0 Hz, 8 H, H8), 4.12 (q, J = 7.2 Hz, 8 H, H8'), 4.20 (s, 8 H, H7'), 6.24 (s, 4 H, H5), 6.55 (s, 2 H, H6), 7.89 (d, J = 5.0 Hz, 2 H, H2), 8.85 (s, 2 H, H1), 8.88 (d, J = 5.0 Hz, 2 H, H3); 13C NMR (126 MHz, DMSO-d₆) δ ppm 13.9, 14.1, 47.7, 48.1, 60.5, 61.1, 112.4, 118.5, 119.5, 122.4, 135.9, 139.2, 142.8,
150.3, 155.5, 164.2, 168.6, 169.0, 170.1; IR (solid state, cm$^{-1}$) 1737 (C=O ester), 1648 (C=O amide); ESI-MS m/z found 1255.4672 [M]$^+$, [C$_{60}$H$_{71}$N$_8$O$_{22}$]$^+$ requires 1255.4682

(5-Nitro-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) methanone), 37d$^{142}$

5-nitroisophthalic acid (300 mg, 1.42 mmol), thionyl chloride (5 mL) and dimethylformamide (1 drop) were heated under reflux for 20 hours. The solvent was then removed in vacuo to yield the acid chloride as a pale yellow solid. The acid chloride was redisolved in anhydrous dichloromethane (15 mL) and 1-aza-18-crown-6 (823 mg, 3.12 mmol) and triethylamine (0.44 mL, 3.1 mmol) added and the resulting mixture stirred, under a nitrogen atmosphere, for 18 hours. The reaction mixture was then concentrated to yield the crude product as a brown solid. This was purified by flash column chromatography (100:40:8 chloroform:acetone:ethanol), to yield the product as a brown solid (760 mg, 1.09 mmol, 76%); $^1$H NMR (300 MHz, CDCl$_3$) δ ppm 3.49 - 3.74 (m, 48 H, H3 - H8), 7.87 (t, J = 1.4 Hz, 1 H, H1), 8.42 (d, J = 1.4 Hz, 2 H, H2); IR (solid state, cm$^{-1}$) 1632 (C=O amide), 1538 (NO$_2$), 1472 (NO$_2$); ESI-MS m/z found 702.3447 [M+H]$^+$, [C$_{32}$H$_{52}$N$_3$O$_{14}$]$^+$ requires 702.3444

(5-Amino-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) methanone, 38d$^{142}$

(5-Nitro-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl) methanone), 37d (650 mg, 0.926 mmol) was dissolved in methanol (10 mL) and the solution degassed. Palladium on activated charcoal (67 mg) was added and the solution degassed again, and then placed under a hydrogen atmosphere and stirred for 16 hours. The solution was filtered twice and concentrated to yield the aniline as a yellow-orange oil (380 mg, 0.566 mmol, 61%); $^1$H NMR (300 MHz, CDCl$_3$) δ ppm 3.49 - 3.93 (m, 48 H, H3 -
H8), 6.73 (s, 2 H, H2), 7.28 (s, 1 H, H1); IR (solid state, cm⁻¹) 3350 (N-H), 1622 (C=O amide); ESI-MS m/z found 694.3531 [M+Na]+, [C₃₂H₅₃N₃O₁₂Na]+ requires 694.3521

**N₄,N₄'-bis(3,5-di(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-16-carbonyl)phenyl-[2,2'-bipyridine]-4,4'-dicarboxamide, 41d**

2,2′-Bipyridine-4,4′-dicarboxylic acid, 40 (50 mg, 0.205 mmol), thionyl chloride (3 mL, 15.4 mmol) and triethylamine (1 drop) were heated under reflux for 18 hours. The reaction mixture was concentrated *in vacuo* to yield the acid chloride as an orange solid. The acid chloride was flushed with nitrogen and used immediately. The acid chloride was redissolved in anhydrous chloroform (5 mL) and added dropwise to a stirred solution of (5-amino-1,3-phenylene)bis((1,4,7,10,13-pentaoxa-16-azacyclooctadecan-16-yl)methanone, 38d (303 mg, 0.451 mmol) in anhydrous chloroform (5 mL) at 0 °C under a nitrogen atmosphere. The solution was then heated under reflux for 48 hours. The reaction mixture was concentrated to yield the crude product as a red-brown solid, which was purified by flash alumina column chromatography (100:40:8 chloroform:acetone:ethanol) to yield the product as a brown solid (278 mg, 0.179 mmol, 87 %); ¹H NMR (300 MHz, CDCl₃) δ ppm 3.40 - 3.62 (m, 80 H, H8 – H12), 3.63 (br. s, 16 H, H7), 7.25 (s, 2 H, H6), 7.89 (d, J = 4.5 Hz, 2 H, H3), 7.97 (br. s, 4 H, H5), 8.81 (d, J = 4.5 Hz, 2 H, H2), 8.93 (s, 2 H, H1); IR (solid state, cm⁻¹) 3490 (N-H), 1678 (C=O amide), 1623 (C=O amide)
Tris (N4,N4'-bis (3,5-di (1,4,7,10,13-pentaaza-16-azacyclooctadecane-16-carbonyl) phenyl-[2,2'-bipyridine]-4,4'-dicarboxamide) ruthenium(II) dinitrate, 42d

N4,N4’-bis(3,5-di(1,4,7,10,13-pentaaza-16-azacyclooctadecane-16-carbonyl)phenyl-[2,2’-bipyridine]-4,4’-dicarboxamide, 41d (294 mg, 0.190 mmol), Ru(II)(DMSO)$_4$Cl$_2$ (28.7 mg, 0.0592 mmol), silver nitrate (20.1 mg, 0.118 mmol) and ethanol (20 mL) were heated under reflux for 7 days. The resulting red solution was then filtered and the filtrate concentrated in vacuo. The resulting red solid was purified by flash alumina column chromatography (1 – 10 % methanol in chloroform) and the red fractions collected. These were then redissolved in water (20 mL) and washed with ether (2 × 20 mL), then extracted with chloroform (3 × 40 mL), and the combined chloroform phases concentrated to yield the product as a red solid (94 mg, 0.019 mmol, 33 %); $^1$H NMR (500 MHz, CDCl$_3$) δ ppm 3.44 - 3.94 (m, 288 H, H7 – H12), 6.71 - 6.80 (m, 18 H, H6 + H4 + H3), 7.39 - 7.51 (m, 18 H, H5 + H2), 8.06 - 8.13 (m, 6 H, H1); IR (solid state, cm$^{-1}$) 3423 (N-H), 1672 (C=O amide), 1627 (C=O amide) (MS was not able to be obtained, presumably due to the multiple ionisation states present due to chelation of many different ions)

Di-tert- butyl (((5-nitroisophthaloyl) bis(azanediyl)) bis (hexane -6,1- diyl)) dicarbamate, 37e

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5-nitroisophthalic acid (200 mg, 0.950 mmol), thionyl chloride (3 mL, 41.4 mmol) and dimethylformamide (1 drop) were heated under reflux for 5 hours. The solvent was then removed in vacuo to yield the acid chloride as a pale yellow solid, which was reacted immediately. The acid chloride was redissolved in anhydrous dichloromethane (15 mL) and N-boc-1,6-diaminohexane (0.47 mL, 2.09 mmol) and triethylamine (0.29 mL, 2.09 mmol) added and the resulting solution stirred for 18 hours. After which time the mixture was washed with 1 M hydrochloric acid (50 mL), saturated sodium hydrogen carbonate solution (50 mL) and brine (50 mL), and dried (sodium sulfate). The solution was concentrated to yield the crude product as a yellow oil. The crude product was purified by flash column chromatography (6:1 to 3:1 ethyl acetate:dichloromethane) to yield the product as a yellow oil (334 mg, 0.550 mmol, 58%); 

\[ ^1H \text{NMR (300 MHz, CDCl}_3 \delta ppm 1.25 - 1.56 (m, 26 H, H6 +H7 + H10), 1.57 - 1.69 (m, 8 H, H5 + H8), 3.16 (d, J = 6.2 Hz, 4 H, H9), 3.46 (q, J = 5.9 Hz, 4 H, H4), 4.85 (br. s, 2 H, NHBoc), 7.28 (br. s, 2 H, H3), 3.46 (app. q, J = 6.0 Hz, 4 H, H4), 8.71 (s, 1 H, H1), 8.89 (s, 2 H, H2);] \n
\[ ^13C \text{NMR (75 MHz, CDCl}_3 \delta ppm 25.6, 25.9 28.4, 29.1, 29.9, 39.9, 79.2, 124.8, 131.0, 136.6, 148.4, 156.6, 164.6; IR (solid state, cm}^{-1} \) 3342 (N-H), 1674 (C=O carbamate), 1645 (C=O amide), 1580 (NO$_2$), 1517 (NO$_2$); ESI-MS m/z found 608.3672 [M+H]$^+$, [C$_{30}$H$_{50}$N$_5$O$_8$]$^+$ requires 608.3659

**Di-tert- butyl (((5- aminoisophthaloyl) bis (azanediyl)) bis (hexane-6,1-diyl)) dicarbamate, 38**

Di-tert-butyl(((5-nitroisophthaloyl)bis(azanediyl))bis(hexane-6,1-diyl))dicarbamate, 37 (1.30 g, 2.14 mmol) in methanol (20 mL) and ethyl acetate (20 mL) was degassed and palladium on activated charcoal added (156 mg) and the solution degassed again. The reaction mixture was then placed under a hydrogen atmosphere and stirred for 18 hours. The reaction mixture was then filtered twice and concentrated to yield the product as a cream solid (1.148 g, 1.99 mmol, 91%); 

\[ ^1H \text{NMR (300 MHz, CDCl}_3 \delta ppm 1.30 - 1.43 (m, 4 H, H5), 1.40 (s, 18 H, H10), 1.43 - 1.52 (m, 4 H, H7), 1.46 - 1.55 (m, 4 H, H6), 1.56 - 1.72 (m, 4 H, H8), 3.16 (d, J = 6.0 Hz, 4 H, H9), 3.46 (app. q, J = 6.0 Hz, 4 H, H4), 8.71 (s, 1 H, H1), 8.89 (s, 2 H, H2);] \n
\[ ^13C \text{NMR (75 MHz, CDCl}_3 \delta ppm 26.0, 26.3, 28.4, 29.3, 29.9, 39.8, 40.1, 79.1, 114.8, 116.8, 135.6, 147.1, 156.4, 167.3; IR (solid state, cm}^{-1} \) 3355 (N-H), 1685 (C=O amide); ESI-MS m/z found 578.3921 [M+H]$^+$, [C$_{30}$H$_{52}$N$_5$O$_6$]$^+$ requires 578.3912

![Chemical Structure](image)

2,2’-Bipyridine-4,4’-dicarboxylic acid, 40 (100 mg, 0.410 mmol) and thionyl chloride (10 mL) were heated under reflux for 18 hours. The reaction mixture was concentrated in vacuo and the acid chloride flushed with nitrogen and reacted immediately. The acid chloride was redissolved in anhydrous chloroform (10 mL) and heated to reflux, under a nitrogen atmosphere. A solution of di-tert-butyl(((5-aminoisophthaloyl)bis(azanediyl))bis(hexane-6,1-diyl))dicarbamate, 38e (426 mg, 0.737 mmol) and anhydrous triethylamine (0.13 mL, 0.90 mmol) in anhydrous chloroform (25 mL) under a nitrogen atmosphere was added dropwise to the refluxing solution and the resulting mixture heated under reflux for 18 hours. The reaction mixture was then concentrated to yield the crude product as a pink solid, which was purified by flash column chromatography (5 – 20 % methanol in dichloromethane) to yield the product as a beige solid (220 mg, 0.161 mmol, 39 %); \(^1\)H NMR (500 MHz, DMSO-d$_6$) \(\delta\) ppm 1.26 - 1.35 (m, 16 H, H10 + H11), 1.40 (s + m, 44 H, H14 + H9/H12), 1.55 (quin., \(J = 7.1\) Hz, 8 H, H9/H12), 2.92 (q, \(J = 6.2\) Hz, 8 H, H8/H13), 3.29 (q, \(J = 7.1\) Hz, 8 H, H8/H13), 6.78 (t, \(J = 5.5\) Hz, 4 H, H7), 8.03 (s, 2 H, H1), 8.05 (d, \(J = 5.2\) Hz, 2 H, H2), 8.40 (s, 2 H, H6), 8.55 (t, \(J = 5.2\) Hz, 4 H, H3), 8.95 - 9.04 (m, 4 H, H5), 10.99 (s, 2 H, H4); ESI-MS m/z found [M+H]$^+$, \([C_{72}H_{107}N_{12}O_{12}]^+\) requires 1363.8030

Tert-butyl N-6-[[3-2-[4-((3,5-bis([(tert-butoxy)carbonyl]amino)hexyl)carbamoyl]phenyl)carbamoyl]pyridin-2-yl]pyridine-4-amido]-5-[[6-((tert-butoxy)carbonyl]amino)hexyl]carbamoyl]phenyl)formamido]hexyl]carbamate, 41e (172 mg, 0.126 mmol), Ru(II)(DMSO)Cl₂ (19 mg, 0.0394 mmol) and silver nitrate (13 mg, 0.0788 mmol) in ethanol (20 mL) were heated under reflux for 7 days. The reaction mixture was then filtered and the filtrate evaporated to yield the product as a red solid (223 mg, 0.0517 mmol, quant.); ¹H NMR (500 MHz, DMSO-<d>₆) δ ppm 1.22 - 1.34 (m, 24 H, H10), 1.36 (s, 108 H, H14), 1.54 (m, 24 H, H8), 2.56 (s, 24 H, H11), 2.91 (q, J = 6.5 Hz, 24 H, H9), 3.29 (d, J = 5.0 Hz, 24 H, H12), 5.77 (s, 24 H, H8/H13), 6.76 (br. s, 24 H, H8/H13), 8.06 (s, 6 H, H6), 8.08 - 8.12 (m, 6 H, H2), 8.12 - 8.22 (m, 6 H, H3), 8.39 (s, 12 H, H5), 8.53 (br. s, 6 H, H1), 9.49 (br. s, 12 H, H7), 11.06 (br. s, 6 H, H4); IR (solid state, cm⁻¹) 3254 (broad) (N-H), 1558 (broad) (C=O amide/carbamate); ESI-MS m/z found 2096.1500 [M]²⁺; [C₂₁₆H₃₁₈N₃₆O₄₂Ru]²⁺ requires 2095.1449
Tris (N1,N3-bis (6-aminohexyl)-5-{2-[4-{{3,5-bis [(6-aminohexyl) carbamoyl] phenyl} carbamoyl} pyridin-2-yl} pyridine-4-amido} benzene-1,3-dicarboxamide) ruthenium(II) dichloride, 35

Tris (tert-butyl N-{6-[[3-{{2-[4-{{3,5-bis][6-{{[(-butoxy)carbonyl] amino} hexyl} carbamoyl] phenyl} carbamoyl} pyridin-2-yl} pyridine-4- amido]-5-[[6-{{[(-butoxy) carbonyl]amino}hexyl}carbamoyl]phenyl]formamido}hexyl}carbamate])} ruthenium(II) dinitrate, 42e (50 mg, 0.012 mmol) in 1 M hydrogen chloride in dioxane (5 mL) was stirred for 18 hours. The solvent was then evaporated and the red solid redissolved in water (10 mL). The solution was then neutralized with 1 M sodium hydroxide solution and the solvent removed. The resulting solid was dialysed (MWCO 0.1 – 0.5 kDa) against pure water to yield the product as a red solid (22 mg, 0.0072 mmol, 60 %); 1H NMR (500 MHz, D2O) δ ppm 1.51 (br. m, 96, H9 – H12), 2.86 (br. m, 48 H, H8 + H13), 7.20 – 8.70 (br. m, 36 H, H1 – H6); IR (solid state, cm⁻¹) 3385 (N-H), 3263 (N-H), 3043 (N-H), 1671 (C=O amide), 1641 (C=O amide) (The many possible ionisation states present prevented mass spectrum analysis)

[2,2'-bipyridine]-4,4'-dicarbohydrazide, 48

4,4'-Dimethylester-2,2'-bipyridine, 47 (100 mg, 0.367 mmol) and hydrazine monohydrate (0.036 mL, 0.73 mmol) in methanol (5 mL) were heated under reflux for 6 hours. The solution was cooled to room temperature, filtered and the precipitate washed with ethanol to yield the product as a very insoluble white powder (66 mg, 0.24 mmol, 67

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%); $^1$H NMR (500 MHz, TFA-d$_x$) δ ppm 8.53 (d, $J = 5.3$ Hz, 2 H, H2), 9.26 (d, $J = 5.3$ Hz, 2 H, H3), 9.33 (s, 2 H, H1); $^{13}$C NMR (126 MHz, TFA-d$_x$) δ ppm 122.1, 125.9, 143.0, 147.0, 147.7, 163.7; IR (solid state, cm$^{-1}$): 3292 (N-H), 3065 (N-H), 1611 (C=O amide) (Poor solubility limited the ability to obtain mass spectra)

**Tris ([2,2'-bipyridine]-4,4'-dicarbohydrazide) dihexafluorophosphate, 43**

Tris (4,4'-dimethylene-2,2'-bipyridine) ruthenium(II) dihexafluorophosphate, 49 (128 mg, 0.0990 mmol) and hydrazine monohydrate (0.053 mL, 1.08 mmol) in methanol (10 mL) were heated under reflux for 5 hours. The hot solution was diluted with ethanol and filtered. The precipitate was redissolved in water, filtered and the filtrate evaporated to yield the product as a red solid (120 mg, 0.093 mmol, 94%); $^1$H NMR (300 MHz, D$_2$O) δ ppm 7.48 - 7.58 (m, 6 H, H2), 7.79 (d, $J = 5.9$ Hz, 6 H, H3), 8.78 (br. s, 6 H, H1); IR (solid state, cm$^{-1}$) 3614 (N-H), 3062 (N-H), 1654 (C=O amide); ESI-MS m/z found 459.1026 [M]$^{2+}$, [C$_{36}$H$_{36}$N$_{18}$O$_6$Ru]$_2$$^+$ requires 459.1054 (this Ru(II)(bpy)$_3$ complex is not stable to O$_2$ and was therefore reacted on immediately)

**Tris (N'-[(1E)-phenylmethylidene] -2-(4-{'N'-(1E)-phenylmethylidene}hydrazinecarbonyl) pyridin-2-yl) pyridine-4-carbohydrazide) ruthenium(II) dihexafluorophosphate, 46a**

To *tris* ([2,2'-bipyridine]-4,4'-dicarbohydrazide) ruthenium(II) dihexafluorophosphate, 43 (94 mg, 0.0778 mmol) in degassed acetonitrile (10 mL) and water (10 mL) under a nitrogen atmosphere was added benzaldehyde (148 mg, 0.891 mmol) and the resulting solution stirred for 30 minutes. The solution was then concentrated *in vacuo*, and the resulting red solid suspended in chloroform (20 mL) and filtered. The precipitate was
reddissolved in acetonitrile and filtered, the filtrate was concentrated to yield the product as a red solid (112 mg, 0.0713, 92 %); 1H NMR (500 MHz, CD$_3$CN) δ ppm 3.64 - 3.72 (br. m, 2 H, H4), 3.72 - 3.80 (br. m, 4 H, H4'), 6.36 (s, 6 H, H1), 7.53 (t, J = 8.0 Hz, 8 H, H7), 7.66 (t, J = 7.1 Hz, 4 H, H7'), 7.77 (dd, J = 7.2, 5.0, 1.6 Hz, 6 H, H2), 8.04 (d, J = 7.1 Hz, 8 H, H6), 8.09 (td, J = 8.0, 1.8 Hz, 4 H, H8), 8.12 (d, J = 8.0 Hz, 4 H, H6'), 8.16 (t, J = 7.1 Hz, 8 H, H8'), 8.66 (d, J = 7.2 Hz, 6 H, H3), 8.74 (d, J = 6.0 Hz, 2 H, H5), 8.84 (d, J = 4.6 Hz, 4 H, H5') (cis and trans isomers of hydrazone seen in 1:2 ratio); IR (solid state, cm$^{-1}$) 3368 (N-H), 1723 (C=O amide), 1654 (C=N); ESI-MS m/z found 723.2003 [M]$^{2+}$, [C$_{78}$H$_{60}$N$_{18}$O$_6$Ru]$^{2+}$ requires 723.1993

Tris (N'- [[(1E)- (2,4- dimethoxyphenyl) methylidene] -2- (4- (N'- [[(1E)- (2,4- dimethoxyphenyl) methylidene] hydrazinecarbonyl] pyridin-2-yl) pyridine-4-carbohydrazide) dihexafluorophosphate, 46b

To tris ([2,2'-bipyridine]-4,4'-dicarbohydrazide) ruthenium(II) dihexafluorophosphate, 43 (94 mg, 0.0778 mmol) in degassed acetonitrile (10 mL) and water (10 mL) under a nitrogen atmosphere was added 2,4-dimethoxy benzaldehyde (148 mg, 0.891 mmol) and stirred for 16 hours. The solution was then concentrated in vacuo, and the resulting red solid suspended in chloroform (20 mL) and filtered. The precipitate was redisolved in acetonitrile and filtered, the filtrate was concentrated to yield the product as a red solid (84 mg, 0.044, 56 %). 1H NMR (500 MHz, CD$_3$CN) δ ppm 3.94 (s, 12 H, H9/H10), 4.01 (s, 12 H, H9/H10), 4.02 - 4.03 (m, 6 H, H9'/H10'), 4.05 (m, 6 H, H9'/H10'), 6.40 - 6.45 (m, 6 H, H4), 6.67 (d, J = 2.3 Hz, 6 H, H1), 6.73 (td, J = 6.5, 2.3 Hz, 6 H, H8), 7.55 (app. d, J = 9.4 Hz, 6 H, H2), 7.63 (dd, J = 6.5, 3.7 Hz, 6 H, H7), 7.74 (d, J = 3.7 Hz, 6 H, H6), 7.86 (d, J = 9.4 Hz, 6 H, H3), 8.87 (app. s, 6 H, H5) (cis and trans hydrazone isomers seen on OMe in 1:2 ratio); IR (solid state, cm$^{-1}$) 1665 (C=O amide), 1594 (C=N); ESI-MS m/z found 903.2673 [M]$^{2+}$, [C$_{90}$H$_{60}$N$_{18}$O$_{18}$Ru]$^{2+}$ requires 903.2627

127
**Tris** (N-[(hydrazinecarbonyl) methyl]-2- (4-[[hydrazinecarbonyl) methyl carbamoyl] pyridin-2-yl]pyridine-4-carboxamide) ruthenium(II) dinitrate, 49

![Chemical Structure](image)

**Tris** (ethyl 2-[[2-[[2-[(2-ethoxy-2-oxoethyl)carbamoyl]pyridin-2-yl]pyridin-4-yl]formamido] acetate) ruthenium(II) dinitrate, 42f (50 mg, 0.0340 mmol) and hydrazine monohydrate (0.02 mL, 0.408 mmol) in ethanol (5 mL) were heated under reflux, under a nitrogen atmosphere, for 18 hours, then filtered. The red precipitate was redissolved in water, filtered and concentrated to yield the product as a red solid (45 mg, 0.034 mmol, 95 %); ¹H NMR (500 MHz, D₂O) δ ppm 4.08 (s, 12 H, H₅), 7.73 (d, J = 5.4 Hz, 6 H, H₂), 7.94 (d, J = 5.4 Hz, 6 H, H₃) 8.97 (s, 6 H, H₁); IR (solid state, cm⁻¹) 3246 (N-H), 3065 (N-H), 1733 (C=O amide), 1650 (C=O amide); ESI-MS found m/z 630.1641 [M]²⁺, [C₄₈H₅₄N₂₄O₁₂Ru]²⁺ requires 630.169


![Chemical Structure](image)

To tris (N- [[(hydrazinecarbonyl) methyl]-2- (4- [[[hydrazinecarbonyl) methyl carbamoyl] pyridin-2-yl]pyridine-4-carboxamide) ruthenium(II) dinitrate, 49 (50 mg, 0.036 mmol) in degassed acetonitrile (10 mL) and water (10 mL) under a nitrogen atmosphere was added benzaldehyde (148 mg, 0.891 mmol) and the resulting solution stirred for 30 minutes. The solution was then concentrated in vacuo, and the resulting red solid suspended in chloroform (20 mL) and filtered, to yield the product as a red precipitate (55 mg, 0.029 mmol, 80 %); ¹H NMR (500 MHz, DMSO-d₆) δ ppm 4.14 (m, 8 H, H₅), 4.56 (br. s, 4 H, H₅’), 7.45 (br. s, 8 H, H⁹), 7.72 (br. s, 4 H, H⁹’), 7.92 (br. s, 12 H, H₁ +
H2), 8.00 - 8.14 (m, 8 H, H8), 8.25 (br. s, 4 H, H8'), 9.39 (br. s, 6 H, H3), 9.50 (br. s, 4 H, H10), 9.70 (d, J = 23.8 Hz, 2 H, H10'), 11.62 (br. s, 4 H, H7), 11.65 - 11.71 (m, 2 H, H7') (cis and trans hydrazone isomers observed in 1:2 ratio); IR (solid state, cm\(^{-1}\)) 3218 (N-H), 1655 (C=O amide), 1541 (C=N); ESI-MS found m/z 894.2639 \([M]^{2+}\), \([C_{90}H_{78}N_{24}O_{12}Ru]^{2+}\) requires 894.2640

**Tris (N-((N'-[(1E)-2,4 dimethoxy phenylmethylidene] hydrazinecarbonyl) methyl)-2-{4-[[N'-[(1E)-2,4 dimethoxy phenylmethylidene] hydrazinecarbonyl] methyl]carbamoyl] pyridin-2-yl}pyridine-4-carboxamide) dinitrate, 50b**

![Chemical Structure](image)

To tris (N-[(hydrazine carbonyl) methyl]-2-{4-[[hydrazine carbonyl] methyl carbamoyl]pyridin-2-yl}pyridine-4-carboxamide) ruthenium(II) dinitrate (50 mg, 0.036 mmol) in degassed acetonitrile (10 mL) and water (10 mL) under a nitrogen atmosphere was added 2,4-dimethoxy benzaldehyde (46 mg, 0.43 mmol) and the resulting solution stirred for 30 minutes. The solution was then concentrated *in vacuo*, and the resulting red solid suspended in dichloromethane (20 mL) and filtered, to yield the product as a red solid (60 mg, 0.026, 73 %); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \(\delta\) ppm 3.84 (m, 36 H, H11 + H12), 4.00 - 4.19 (m, 4 H, H5), 4.52 (br. s, 8 H, H5'), 6.48 - 6.74 (m, 12 H, H8 + H9), 7.56 - 7.82 (m, 6 H, H2), 7.91 (br. s, 6 H, H1), 8.00 - 8.17 (br. s, 6 H, H3), 8.27 (br. s, 4 H, H9), 8.41 - 8.56 (m, 2 H, H9'), 9.26 - 9.53 (m, 6 H, H10), 11.28 - 11.57 (m, 6 H, H7) (cis and trans hydrazone isomers seen in 1:2 ratio); IR (solid state, cm\(^{-1}\)) 3215 (N-H), 1659 (C=O amide), 1600 (C=N); ESI-MS found m/z 1074.3298 [M]^{2+}, \([C_{102}H_{102}N_{24}O_{24}Ru]^{2+}\) requires 1074.3271
Tris (N-[(N'-[(1E)-4 hydroxy phenylmethylidene]hydrazinecarbonyl]methyl)-2-{4-[(N'-[(1E)-4 hydroxy phenyl methylidene] hydrazine carbonyl}methyl) carbamoyl] pyridin-2-yl}pyridine-4-carboxamide) dinitrate, 50d

To tris (N-[hydrazine carbonyl] methyl)-2-{4-[[hydrazine carbonyl] methyl] carbamoyl} pyridin-2-yl]pyridine-4-carboxamide) ruthenium(II) dinitrate (10 mg, 0.0072 mmol) in degassed acetonitrile (5 mL) and water (5 mL) under a nitrogen atmosphere was added 4-hydroxy benzaldehyde (20 mg, 0.16 mmol) and the resulting solution stirred for 30 minutes. The solution was then concentrated in vacuo, and the resulting red solid suspended in dichloromethane (20 mL) and filtered, to yield the product as a red solid (16 mg, 0.080 mmol, quant.); 1H NMR (500 MHz, DMSO-d6) δ ppm 4.04 (br. s, 2 H, H5), 4.48 (br. s, 10 H, H5'), 6.80 (br. s, 10 H, H9), 6.93 (br. s, 2 H, H9'), 7.45 (br. s, 10 H, H8), 7.74 (br. s, 2 H, H8'), 7.88 (br. s, 6 H, H2), 8.10 (br. s, 6 H, H3), 9.28 (br. s, 6 H, H1), 9.75 (br. s, 1 H, H7), 9.90 (br. s, 5 H, H7'), 11.37 (br. s, 6 H, H4) (cis and trans hydrazone isomers seen in a 1:5 ratio); IR (solid state, cm⁻¹) 3213 (N-H), 3075 (O-H), 1654 (C=O amide), 1603 (C=N); ESI-MS found m/z 972.2506 [M]²⁺, [C₉₈H₇₅N₂₄O₁₈Ru]²⁺ requires 1074.3271

Tris (methyl (2S)-3-(tert- butoxy)- 2-{2-(4-{[(2S)-3-(tert- butoxy)- 1-methoxy- 1-oxopropan-2-yl} carbamoyl} pyridin-2-yl) pyridin-4-yl]formamido} propanoate) ruthenium(II) dichloride, 41j

Tris (2,2'-bipyridine-4,4'-dicarboxylic acid) ruthenium(II) dichloride, 29 (120 mg, 0.133 mmol) was heated under reflux in thionyl chloride (20 mL) and dimethylformamide (1 drop) for 6 hours. The reaction mixture was concentrated in vacuo and the acid chloride flushed with nitrogen and used immediately. The resulting acid chloride was resuspended
in anhydrous chloroform (30 mL) and heated to reflux. **O-tert-Butyl-L-serine methyl ester hydrochloride salt** (252 mg, 1.19 mmol) and anhydrous disopropylethylamine (0.41 mL, 2.4 mmol) were added to the refluxing solution and the reaction mixture heated under reflux for 18 hours under a nitrogen atmosphere. The reaction mixture was cooled to room temperature and washed with saturated sodium hydrogen carbonate solution (30 mL), 1 M hydrochloric acid (30 mL) and brine (30 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a red solid. This was purified by flash column chromatography (10 % methanol in dichloromethane) to yield the product as a red solid (108 mg, 0.0584 mmol, 44 %); \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ ppm, 1.17 (s, 54 H, H8), 3.74 (s, 18 H, H7), 3.80 (dd, \(J = 9.2, 4.4 \text{ Hz}, 6 \text{ H, H6'}\)), 3.93 (dd, \(J = 9.2, 4.4 \text{ Hz}, 6 \text{ H, H6}\)), 4.86 (dt, \(J = 7.4, 4.4 \text{ Hz}, 6 \text{ H, H5}\)), 7.82 (dd, \(J = 12.3, 5.4 \text{ Hz}, 6 \text{ H, H2}\)), 7.92 (d, \(J = 5.4 \text{ Hz}, 6 \text{ H, H1}\)), 8.78 (d, \(J = 7.4 \text{ Hz}, 6 \text{ H, H4}\)), 9.36 (d, \(J = 5.4 \text{ Hz}, 6 \text{ H, H3}\)); IR (solid state, cm\(^{-1}\)) 3243 (N-H), 1739 (C=O tester), 1662 (C=O amide); ESI-MS \(m/z\) found 888.3430 [M]\(^{2+}\), [C\(_{84}\)H\(_{114}\)N\(_{12}\)O\(_{24}\)Ru]\(^{2+}\) requires 888.3556

**Tris** (methyl (2S)-3-(hydroxy)-2-{{2-4-{{[(2S)-3-(hydroxy)-1-methoxy-1-oxopropan-2-yl]carbamoyl} pyridin-2-yl} pyridin-4-yl} formamido} propanoate) ruthenium(II) ditrifluoroacetate, 58a

\[
\begin{align*}
\text{Ru(II)Cl}_2 & \quad \text{MeOD} \\
\text{H} & \quad \text{N} \\
\text{O} & \quad \text{C} \\
\text{O} & \quad \text{COOMe} \\
\text{N} & \quad \text{H} \\
\text{O} & \quad \text{MeOOC} \\
\text{H} & \quad \text{HO} \\
\text{H} & \quad \text{OH} \\
\text{H} & \quad \text{OH} \\
\end{align*}
\]

**Tris** (methyl (2S)-3-{{(tert-butoxy)-2-{{2-4-{{[(2S)-3-{{(tert-butoxy)-1-methoxy-1-oxopropan-2-yl] carbamoyl} pyridin-2-yl} pyridin-4-yl} formamido} propanoate) ruthenium(II) dinitrate, 41j (25 mg, 0.015 mmol) in trifluoroacetic acid (4.5 mL), water (0.4 mL) and triisopropylsilane (0.1 mL) was stirred for 6 hours. The reaction mixture was then concentrated to yield the product as a red solid (24 mg, 0.014 mmol, 96 %); \(^1\)H NMR (500 MHz, MeOD) δ ppm 2.70 (s, 18 H, H7), 4.00 (dd, \(J = 11.5, 3.8 \text{ Hz}, 6 \text{ H, H6'}\)), 4.06 (dd, \(J = 11.5, 5.5 \text{ Hz}, 6 \text{ H, H6}\)), 4.76 - 4.87 (m, 6 H, H5), 7.95 (d, \(J = 5.8 \text{ Hz}, 6 \text{ H, H2}\)), 8.09 (d, \(J = 5.8 \text{ Hz}, 6 \text{ H, H3}\)), 9.27 (s, 6 H, H1); IR (solid state, cm\(^{-1}\)) 3290 (O-H), 3071 (N-H), 1733 (C=O ester), 1656 (C=O amide); ESI-HRMS found \(m/z\) 720.1714 [M]\(^{2+}\), [C\(_{60}\)H\(_{66}\)N\(_{12}\)O\(_{24}\)Ru]\(^{2+}\) requires 720.1678
Tris (N4, N4’- bis (6-boc aminohexyl) -2,2’- bipyridine- 4,4’- dicarboxamide) ruthenium(II) dichloride, 41i

Tris (2,2’-bipyridine-4,4’-dicarboxylic acid) ruthenium(II) dichloride, 29 (114 mg, 0.125 mmol) was heated under reflux in thionyl chloride (30 mL) and dimethylformamide (1 drop) for 6 hours. The solvent was removed in vacuo and the resulting red acid chloride flushed with nitrogen and used immediately. The acid chloride was resuspended in anhydrous chloroform (30 mL) and heated to reflux, under a nitrogen atmosphere. N-Boc-1,6-diamino hexane (0.25 mL, 1.1 mmol) and anhydrous disopropylethylamine (0.39 mL, 2.3 mmol) were added, and the resulting solution heated under reflux for 16 hours. The reaction mixture was then allowed to cool to room temperature, and the reaction mixture quenched with saturated sodium hydrogen carbonate solution (30 mL). The aqueous layer was removed and the organic phase washed with 1 M hydrochloric acid (30 mL) and brine (30 mL). The organic phase was dried (sodium sulfate) and concentrated in order to yield the crude product as a red solid. This was purified by flash column chromatography (10 % methanol in dichloromethane) to yield the product as a red solid (95 mg, 0.045 mmol, 36 %); 1H NMR (500 MHz, CDCl3) δ ppm 1.34 (br. d, J = 5.2 Hz, 12 H, H7/H8), 1.39 (s, 54 H, H11), 1.44 - 3.47 (br. s, 12 H, H7/H8), 1.53 (m, 12 H, H6/H9), 1.69 (br. s, 24 H, H6/H9 + H5/H10), 3.09 (br. s, 12 H, H5/H10), 4.77 (br. s, 6 H, NHBoc), 7.66 (br. s, 6 H, H2), 8.06 (br. s, 6 H, H1), 8.97 (br. s, 6 H, H3), 10.02 (br. s, 6 H, H4); IR (solid state, cm⁻¹) 3291 (N-H), 1657 (C=O amide); ESI-HRMS found m/z 1011.5489 [M]²⁺, [C₁₀₂H₁₅₂aN₁₈O₁₈Ru]²⁺ requires 1011.5444
**Tris** (N4,N4′-bis (6-aminohexyl) -2,2'-bipyridine-4,4'-dicarboxamide) ruthenium(II) dichloride, 81

*Tris* (N4,N4′-bis(6-Boc aminohexyl)-2,2'-bipyridine-4,4'-dicarboxamide) ruthenium(II) dichloride (20 mg, 0.0095 mmol) was stirred in 1 M hydrogen chloride in dioxane (5 mL) and water (0.5 mL) for 2 hours. The resulting mixture was concentrated and redissolved in water (10 mL). The solution was neutralised by addition of 1 M sodium hydroxide solution. The neutral solution was concentrated to ~2 mL and the resulting solution dialysed (MWCO 0.1 - 0.5 kDa) against pure water to yield the product as a red solid (14 mg, 0.0094 mmol, 98 %); 1H NMR (500 MHz, D2O) δ ppm 1.37 (br. s, 24 H, H7 + H8), 1.62 (br. s, 24 H, H6 + H9), 2.94 (t, J = 7.5 Hz, 12 H, H5/H10), 3.38 (t, J = 6.8 Hz, 12 H, H5/H10), 7.67 (d, J = 5.8 Hz, 6 H, H3), 7.90 (d, J = 5.8 Hz, 6 H, H2), 8.89 (s, 6 H, H1); IR (solid state, cm⁻¹) 3386 (N-H), 3255 (N-H), 1717 (C=O amide); ESI-MS m/z found 711.3884 [M]²⁺, [C72H108N18O6Ru]²⁺ requires 711.3871

**Tris** (4-tert-butyl 1-methyl (2S)-2-[[4'-[[2S]-4-(tert-butoxy)-1-methoxy-1,4-dioxobutan-2-yl] carbamoyl]- [2,2'-bipyridin]-4-yl] formamido] butanedioate) ruthenium(II) dichloride, 41k

*Tris* (2,2'-bipyridine-4,4'-dicarboxylic acid) ruthenium(II) dichloride (50 mg, 0.055 mmol) was heated under reflux in thionyl chloride (20 mL) and dimethylformamide (1 drop) for 6 hours. The reaction mixture was concentrated in vacuo and the acid chloride flushed with nitrogen. The resulting acid chloride was resuspended in anhydrous
Tert-butyl 2-(((9H-fluoren-9-yl) methoxy) carbamylamino) -3- tert-butoxypropanoyl) hydrazine carboxylate, 61a

Fmoc-Ser (OtBu) COOH (5.00 g, 13.0 mmol), tert-butyl carbazate (5.17 g, 39.1 mmol), HATU (5.44 g, 14.3 mmol) and diisopropylethylamine (9.02 mL, 52.0 mmol) were stirred in anhydrous dimethylformamide (20 mL) under a nitrogen atmosphere for 18 hours. The solution was then diluted with ethyl acetate (300 mL) and washed with saturated sodium hydrogen carbonate solution (200 mL), 1 M hydrochloric acid (200 mL) and brine (3 x 500 mL) to yield the crude product as an off-white solid. This was purified by flash column chromatography (20 % ethyl acetate in dichloromethane) to yield the product as a white solid (4.32 g, 8.68 mmol, 67 %); 1H NMR (500 MHz, CDCl3) δ ppm 1.24 (s, 9 H, H1/H5), 1.50 (s, 9 H, H1/H5), 3.48 (m, 1 H, H2+H3), 3.74 (q, J = 6.5 Hz, 2 H, H4), 4.25 (t, J = 7.0 Hz, 1 H, H7), 4.44 (d, J = 7.0 Hz, 2 H, H6), 5.70 (br. s, 1 H, NH), 6.50 (br. s, 1 H, NH), 7.34 (t, J = 7.6 Hz, 2 H, Fmoc), 7.43 (t, J = 7.6 Hz, 2 H, Fmoc), 7.62 (dd, J = 7.6, 3.0 Hz, 2 H, Fmoc), 7.79 (d, J = 7.6 Hz, 2 H, Fmoc), 8.42 (br. s, 1 H, NH); 13C NMR (75 MHz, DMSO-d6) δ ppm 27.2, 28.0, 46.6, 54.2, 61.4, 61.8, 65.8, 72.9, 120.0, 127.0, 127.6, 125.4, 140.7, 143.7, 143.8, 155.0, 155.8; IR (solid state, cm⁻¹) 3297 (N-H), 3256 (N-H), 1714 (C=O carbamate), 1688 (C=O amide); ESI-MS m/z found 520.2422 [M+Na]+, [C27H35N3O6Na]+ requires 520.2424

chloroform (30 mL) and H-Asp(OtBu) OMe. hydrogen chloride salt (199 mg, 0.497 mmol) and anhydrous diisopropylethylamine (0.17 mL, 0.99 mmol) were added and the reaction mixture heated under reflux under a nitrogen atmosphere for 18 hours. The resulting solution was then cooled to room temperature and washed with saturated sodium hydrogen carbonate solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (50 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a red solid. This was purified by flash column chromatography (10 % methanol in dichloromethane) to yield the product as a red solid (30 mg, 0.015 mmol, 27 %); 1H NMR (500 MHz, CDCl3) δ ppm 1.36 - 1.52 (m, 60 H, H6 + H8), 1.59 - 1.71 (m, 6 H, H6'), 3.72 (s, 18 H, H7), 5.07 (m, 6 H, H5), 7.74 (br. s, 6 H, H3), 7.98 (br. s, 6 H, H2), 9.24 (br. s, 6 H, H1), 9.87 - 10.23 (m, 6 H, H4); IR (solid state, cm⁻¹) 3055 (N-H), 1723 (C=O ester), 1666 (C=O amide) ESI-MS m/z found 972.3447 [M+2]+, [C90H114N12O30Ru] requires 972.3404
Tert-butyl 2-(2-amino-3-tert-butoxypropanoyl)hydrazinecarboxylate, 62a

![Chemical Structure](image)

Tert-butyl 2-(([(9H-fluoren-9-yl)methoxy]carbonylamino)-3-tert-butoxypropanoyl)hydrazine carboxylate, 61a (1.00 g, 2.00 mmol) in 20 % diethylamine in acetonitrile was stirred for 16 hours. The reaction mixture was concentrated, and redissolved in a minimal amount of ethyl acetate and precipitated with hexane. The suspension was filtered through a celite pad and washed with hexane. The celite pad was then washed with dichloromethane and methanol, and this filtrate concentrated to yield the product as an off-white waxy solid (490 mg, 1.78 mmol, 88 %); \(^{1}H\) NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 1.18 - 1.28 (m, 9 H, H1/H5), 1.50 (s, 9 H, H1/H5), 1.66 - 1.99 (m, 4 H, H6+H2+ NHBoc), 3.55 (dd, \(J = 4.7, 3.1\) Hz, 1 H, H3), 3.59 - 3.68 (m, 2 H, H4/H4'); \(^{13}C\) NMR (101 MHz, MeOD) \(\delta\) ppm 26.3, 27.2, 42.1, 63.2, 73.4, 80.6, 156.3, 172.5; IR (solid state, cm\(^{-1}\)) 3368 (N-H), 3242 (N-H), 1720 (C=O carbamate), 1692 (C=O amide); ESI-MS \(m/z\) found 276.1923 [M+H]\(^+\), \([C_{12}H_{26}N_{4}O_4]\)^+ requires 276.1923

N4,N4'- bis [(1S)-2- (tert- butoxy) -1- {N'- [tert- butoxy] carbonyl} hydrazinecarbonyl] ethyl]-[2,2'-bipyridine]-4,4'-dicarboxamide, 68

![Chemical Structure](image)

2,2’-Bipyridine-4,4’-dicarboxylic acid, 40 (241 mg, 0.988 mmol) was heated under reflux in thionyl chloride (30 mL) for 18 hours, and the reaction mixture then concentrated \textit{in vacuo}. The resultant acid chloride was flushed with nitrogen and used immediately. The acid chloride was redissolved in anhydrous chloroform (30 mL) and heated to reflux, under a nitrogen atmosphere. Tert-butyl 2-(2-amino-3-tert-butoxypropanoyl) hydrazinecarboxylate, 62a (598 mg, 2.17 mmol) and anhydrous diisopropylethylamine (0.76 mL, 4.34 mmol) was added to the refluxing solution and the mixture heated under reflux for 18 hours. The solution was cooled to room temperature and washed with 1 M hydrochloric acid (50 mL) and brine (50 mL). The organic phase was dried (sodium sulfate), and concentrated to yield the crude product as a pink solid. This
was purified by flash column chromatography (5% methanol in dichloromethane) to yield the product as a beige solid (122 mg, 0.178 mmol, 16%); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 1.30 (s, 18 H, H8/H9), 1.48 (s, 18 H, H8/H9), 3.67 (t, \(J = 8.5\) Hz, 2 H, H6), 3.92 (dd, \(J = 8.5, 4.8\) Hz, 2 H, H6'), 4.85 (br. s, 2 H, H5), 6.96 - 7.12 (br. s, 2 H, NHBoc), 7.67 (d, \(J = 4.4\) Hz, 2 H, H3), 7.78 - 7.91 (m, 2 H, H7), 8.53 (br. s, 2 H, H1), 8.70 (d, \(J = 4.4\) Hz, 2 H, H2), 9.12 (br. s, 2 H, H4); IR (solid state, cm\(^{-1}\)) 3279 (N-H), 1702 (C=O carbamate), 1651 (C=O amide); ESI-MS m/z found 759.3905 [M+H]\(^+\), [C\(_{36}\)H\(_{55}\)N\(_8\)O\(_{10}\)]\(^+\) requires 759.4041

\((S)-\text{tert-butyl} \ 2-(2-(((9H-fluoren-9-yl) methoxy) carbamylamino)-4-\text{tert-butoxy-4-oxobutanoyl})\text{hydrazinecarboxylate, 61b}\)

\(\text{Fmoc- Asp(OtBu)COOH (1.50 g, 3.65 mmol), \text{tert-butyl carbazate (1.45 g, 10.9 mmol), HATU (1.53 g, 4.00 mmol and diisopropylethylamine (1.27 mL, 7.30 mmol) in anhydrous dimethylformamide (10 mL) were stirred for 16 hours under a nitrogen atmosphere. The solution was then diluted with ethyl acetate (100 mL) and washed with saturated sodium hydrogen carbonate solution (100 mL), 1 M hydrochloric acid (100 mL), brine (3 \times 200 mL) and ammonium hydroxide solution (100 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the product as a white solid (2.02 g, 3.85 mmol, quant.);}\)

\(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 1.48 (s, 9 H, H1/H5), 1.49 (s, 9 H, H1/H5), 2.69 (m, 1 H, H4), 2.91 (m, 1 H, H4'), 4.25 (t, \(J = 7.5\) Hz, 1 H, H7), 4.41 - 4.54 (m, 1 H, H3) 4.57 - 4.68 (m, 1 H, NH) 5.98 (d, \(J = 7.5\) Hz, 1 H, H6), 6.40 (br. s, 1 H, NH), 7.34 (t, \(J = 7.5\) Hz, 2 H, Fmoc) 7.43 (t, \(J = 7.5\) Hz, 2 H, Fmoc), 7.61 (dd, \(J = 7.5, 3.7\) Hz, 2 H, Fmoc), 7.79 (d, \(J = 7.5\) Hz, 2 H, Fmoc), 8.22 (br. s, 1 H, NH); \(^{13}\)C NMR (75 MHz, DMSO-d\(_6\)) \(\delta\) ppm 27.4, 27.7, 28.0, 31.3, 46.6, 52.3, 65.7, 79.7, 120.1, 125.3, 127.1, 127.6, 140.7, 143.8, 155.1, 155.8, 171.0, 171.6; IR (solid state, cm\(^{-1}\)) 3285 (N-H), 1695 (C=O amide); ESI-MS m/z found 548.2265 [M+Na]\(^+\), [C\(_{28}\)H\(_{35}\)N\(_3\)O\(_7\)Na]\(^+\) requires 548.2373

\((S)-\text{tert-butyl} \ 2-(2-amino-4-\text{tert-butoxy-4-oxobutanoyl}) \text{hydrazinecarboxylate 62b}\)
(S)-tert-butyl 2-((9H-fluoren-9-yl methoxy) carbonyl amino)-4-tert-butoxy-4-oxobutanoyl) hydrazine carboxylate, 61b (1.85 g, 3.52 mmol) in 20% diethylamine in acetonitrile (50 mL) was stirred for 16 hours. The reaction solution was then concentrated and the resulting residue redissolved in a minimal amount of ethyl acetate, and precipitated by addition of hexane. The slurry was then filtered through celite. The celite pad was then washed with dichloromethane and methanol and this filtrate concentrated to yield the product as an off-white waxy solid (1.03 g, 3.40 mmol, 96%); 1H NMR (500 MHz, CDCl3) δ ppm 1.48 (s, 9 H, H1/H2), 1.50 (s, 9 H, H1/H2), 2.59 (dd, J = 16.7, 8.1 Hz, 1 H, H4), 2.82 (dd, J = 16.7, 3.7 Hz, 1 H, H4'), 3.16 (br. s, 1 H, H3), 3.91 (br. s, 2 H, H6), 6.38 (br. s, 1 H, H2); 13C NMR (101 MHz, MeOD) δ ppm 23.5, 25.8, 27.0, 36.7, 76.7, 81.1, 155.4, 170.4, 172.3; IR (solid state, cm⁻¹) 3274 (N-H), 1707 (C=O ester); ESI-MS m/z found 326.1685 [M+Na⁺]⁺, [C13H25N3O5Na]⁺ requires 326.1686

(S)-tert-butyl 2-((9H-fluoren-9-yl) methoxy) carbonylamino)-5-tert-butoxy-5-oxopentanoyl) hydrazinecarboxylate, 61c

Fmoc Glu(OtBu)COOH (3.00 g, 7.05 mmol), HATU (2.95 g, 7.76 mmol), tert-butyl carbazate (2.80 g, 21.2 mmol) and diisopropylethylamine (2.46 mL, 14.1 mmol) in anhydrous dimethylformamide (20 mL) were stirred under a nitrogen atmosphere for 18 hours. The solution was then diluted with ethyl acetate (100 mL), and washed with saturated sodium hydrogen carbonate solution (100 mL), 1 M hydrochloric acid (100 mL), brine (3 × 200 mL) and ammonium hydroxide solution (100 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the product as a white solid (3.85 g, 7.13 mmol, 92%); 1H NMR (500 MHz, CDCl3) δ ppm 1.47 (s, 9 H, H1/1Bu), 1.48 (s, 9 H, H1/1Bu), 2.01 (dq, J = 14.0, 8.0 Hz, 1 H, H4), 2.15 (m, 1 H, H4'), 2.46 (m, 2 H, H5), 4.22 (t, J = 6.8 Hz, 1 H, H7), 4.32 (m, 1 H, H3), 4.40 (d, J = 6.8 Hz, 2 H, H6), 5.92 (br. s, 1 H, NH), 6.63 (br. s, 1 H, NH), 7.32 (t, J = 7.2 Hz, 2 H, Fmoc), 7.41 (t, J = 7.2 Hz, 2 H, Fmoc), 7.61 (dd, J = 7.2, 4.0 Hz, 2 H, Fmoc), 7.78 (d, J = 7.2 Hz, 2 H, Fmoc), 8.45 (m, 1 H, NH); 13C NMR (75 MHz, DMSO-d6) δ ppm 27.7, 28.0, 31.3, 46.6, 52.3, 65.7, 79.1, 79.7, 120.0, 125.3, 127.0, 127.6, 140.7, 143.7, 143.9, 155.1, 155.8, 170.3, 171.0; IR (solid state, cm⁻¹) 3274 (N-H), 1692 (C=O amide); ESI-MS m/z found 562.2529 [M+Na⁺], [C29H37N3O7Na]⁺ requires 562.2529
(S)-tert-butyl 2-(2-amino-5-tert-butoxy-5-oxopentanoyl)hydrazinecarboxylate, 62c

(S)-tert-butyl 2-((9H-fluoren-9-yl) methoxy) carbonylamino) -5- tert- butoxy-5-oxopentanoyl) hydrazinecarboxylate, 61c (650 mg, 1.20 mmol) in 20 % diethylamine in acetonitrile (50 mL) was stirred for 16 hours. The reaction solution was then concentrated and the resulting residue redissolved in a minimal amount of ethyl acetate, and precipitated by addition of hexane. The slurry was then filtered through celite. The celite pad was then washed with dichloromethane and methanol and this filtrate concentrated to yield the product as an off-white waxy solid (257 mg, 0.811 mmol, 68 %); 1H NMR (500 MHz, CDCl3) δ ppm 1.45 (s, 9 H, H1/tBu), 1.48 (s, 9 H, H1/tBu), 1.85 (dq, J = 14.0, 7.0 Hz, 1 H, H4), 2.10 (dq, J = 14.0, 7.0 Hz, 1 H, H4'), 2.41 (app. t, J = 7.0 Hz, 2 H, H5), 3.51 (app. t, J = 7.0 Hz, 1 H, H3); 13C NMR (75 MHz, DMSO-d6) δ ppm 27.7, 28.0, 30.6, 31.3, 48.5, 52.7, 79.4, 155.2, 172.2, 174.4; IR (solid state, cm⁻¹) 3293 (N-H), 1716 (C=O tester), 1595 (C=O amide); ESI-MS m/z found 318.2036 [M+H]+, [C14H27N3O5]⁺ requires 318.2029


Tetracarboxyphenyl porphyrin (100 mg, 0.126 mmol), PyBOP (394 mg, 0.757 mmol), diisopropylethylamine (0.26 mL, 1.51 mmol) and ethyl glycine hydrochloride salt (106 mg, 0.757 mmol) in anhydrous dimethylformamide (5 mL) were stirred for 16 hours. Methylisocyanate polystyrene resin (0.340g, 0.063 mmol, 200-400 mesh 1.8 mmol/g) was
then added and the reaction mixture stirred for a further 3 hours. The solution was then
diluted with dichloromethane (50 mL) and washed successively with saturated sodium
carbonate solution (100 mL), 1 M hydrochloric acid (100 mL) and brine (100 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude
product as a purple solid. This was purified by flash column chromatography (3:7 ethyl
acetate:dichloromethane) to yield the product as a purple solid (45 mg, 0.0397 mmol, 32 %); 1H NMR (500 MHz, DMSO-d6) δ ppm 1.31 (t, J = 6.8 Hz, 12 H, Et CH3), 4.19 (d, J = 5.9 Hz, 8 H, H6), 4.24 (q, J = 6.8 Hz, 8 H, Et CH2), 8.34 (d, J = 8.5 Hz, 8 H, H4), 8.38 (d, J = 8.5 Hz, 8 H, H3), 8.82 - 8.94 (s, 4 H, H2), 9.34 (t, J = 5.9 Hz, 4 H, H5); IR (solid state, cm⁻¹) 3272 (N-H), 1759 (C=O tester), 1638 (C=O amide); ESI-HRMS found m/z 1132.4295 [M]+, [C₆₄H₆₆N₈O₁₂]+ requires 1132.4331

Methyl (2S)-2-((4-[7,12-bis(4-((2R)-3-(tert-butoxy)-1-methoxy-1-oxopropan-2-yl) carbamoyl) phenyl)-17- (4-((2S)-3-(tert-butoxy)-1-methoxy-1-oxopropan-2-yl) carbamoyl) phenyl)-21,22,23,24- tetraazapentacyplo [16.2.1.1]³,6,1⁸,11,1³,1⁶] tetracosa-1,3 (24), 4, 6, 8, 10, 12, 14, 16 (22), 17,19-undecaen-2-yl) phenyl formamido)-3-(tert-butoxy) propanoate, 76

Tetracarboxy phenyl porphyrin (50 mg, 0.067 mmol), PyBOP (197 mg, 0.402 mmol), 0-tert-butyl-L-serine methyl ester hydrochloride (85 mg, 0.402 mmol), and diisopropylethylamine (0.14 mL, 0.40 mmol) in anhydrous dimethylformamide (5 mL) were stirred under a nitrogen atmosphere for 18 hours. The reaction mixture was then
dissolved in ethyl acetate (50 mL) and washed with saturated sodium hydrogen carbonate
solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (3 × 100 mL). The organic
phase was dried (sodium sulfate) and concentrated to yield the product as a purple solid
(97 mg, 0.076 mmol, quant.); 1H NMR (500 MHz, CDCl3) δ ppm 1.18 (s, 36 H, H9), 3.77 -
3.82 (m, 8 H, H8 + H7), 3.98 (dd, J = 9.2, 2.7 Hz, 4 H, H7'), 5.05 (dt, J = 8.3, 2.7 Hz, 4 H, H6),
7.27 (d, J = 8.3 Hz, 4 H, H5), 8.17 (d, J = 8.0 Hz, 8 H, H4), 8.22 - 8.29 (d, J = 8.0 Hz, 8 H, H3),
8.78 (s, 8 H, H2); 13C NMR (75 MHz, CDCl3) δ ppm 27.5, 46.3, 52.6, 62.2, 73.7, 119.4, 125.7,
128.5, 131.2, 133.6, 134.5, 167.2, 171.2; IR (solid state, cm⁻¹) 3314 (N-H), 1741 (C=O ester),
1656 (C=O amide); ESI-MS m/z found 1419.6575 [M+H]^+; [C₈₀H₉₀N₈O₁₆]^+ requires 1419.6553

4-[7,12-Bis{4-[(1R)-2-(tert-butoxy)-1-{N'-(tert-butoxy) carbonyl] hydrazine carbonyl} ethyl] carbamoyl] phenyl]-17-{4-[(1S)-2-(tert-butoxy) carbonyl] hydrazinecarbonyl ethyl] carbamoyl} phenyl]-21,22,23,24-tetraazapentacyclo[16.2.1.1³,⁶.1⁸,¹¹.1¹³,¹⁶]tetracosa-1,3(24), 4,6,8,10,12,14,16(22),
17,19-undecaen-2-yl]-N-[(1S)-2-(tert-butoxy)-1-{N'-(tert-butoxy)carbonyl] hydrazine carbonyl] ethyl]benzamide, 78a

Tetracarboxyphenyl porphyrin (100 mg, 0.126 mmol), PyBOP (395 mg, 0.759 mmol),
 tert-butyl 2-(2-amino-3-tert-butoxypropanoyl)hydrazinecarboxylate, 62a (209 mg, 0.759
mmol) and diisopropylethylamine (0.26 mL, 1.5 mmol) in anhydrous dimethylformamide
(5 mL) were stirred under a nitrogen atmosphere for 18 hours. The reaction mixture was
then diluted with ethyl acetate (50 mL) and washed with saturated sodium hydrogen
carbonate solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (3 x 50 mL). The
organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a
purple solid. This was purified by flash column chromatography (1:1 ethyl acetate:dichloromethane then ethyl acetate) to yield the product as a purple solid (142
mg, 0.078 mmol, 62%); ¹H NMR (500 MHz, CDCl₃) δ ppm 1.29 (s, 36 H, H8/H9), 1.45 (m,
36 H, H8/H9), 3.51 - 3.63 (m, 8 H, H7 + H7'), 4.07 (dt, J = 8.3, 4.7 Hz, 4 H, H6), 4.83 (br. s, 4
H, NH), 6.40 - 6.57 (m, 4 H, NH), 8.15 (d, J = 7.5 Hz, 8 H, H4), 8.21 (d, J = 7.5 Hz, 8 H, H3),
8.40 (d, J = 8.3 Hz, 4 H, H5), 8.73 (br. s, 8 H, H2); 13C NMR (101 MHz, MeOD) δ ppm 26.8, 27.5, 38.4, 53.3, 61.6, 74.0, 80.8, 106.6, 119.3, 125.8, 133.4, 134.4, 145.2, 156.2, 168.4, 170.8; IR (solid state, cm⁻¹) 3251 (N-H), 1696 (C=O carbamate), 1645 (C=O amide); ESI-MS m/z found 1820.9402 [M+2H]^+; [C₉₆H₁₂₄N₁₆O₂₀]^+ requires 1820.9178

4- [7,12-Bis {4-[(1R)-2-[(tert-butoxy)carbonyl]hydrazinecarbonyl] ethyl} carbamoyl] phenyl]-17-((4-[[1S]-2-hydroxy-1-(N'-[(1E)-phenylmethylidene] hydrazinecarbonyl] ethyl) carbamoyl) phenyl)-21,22,23,24-tetraazapentacyclo [16.2.1.1³,⁶.1⁸,¹¹.1¹³,¹⁶] tetracosa-1,3(24),4,6,8,10,12,14,16(22), 17,19- undecaen-2-yl] -N-[(1S)-2- hydroxy-1- [N'- [(1E)-phenylmethylidene] hydrazinecarbonyl] ethyl]benzamide, 79a

4-[7,12-bis{4-[[{(1R)-2-(tert-butoxy)} carbonyl] hydrazinecarbonyl] ethyl} carbamoyl] phenyl]-17-((4- [[[1S]-2- (tert-butoxy) -1-{N'-[(tert-butoxy) carbonyl] hydrazine carbonyl] ethyl} carbamoyl]phenyl)- 21,22,23,24-tetraazapentacyclo [16.2.1. 1³,⁶,¹⁸,¹¹,¹³,¹₆] tetracosa-1,3(24),4,6,8,10,12,14,16(22), 17,19-undecaen-2-yl]-N-[[1S]-2- (tert-butoxy)-1-{N'-[(tert-butoxy) carbonyl] hydrazinecarbonyl] ethyl]benzamide, 78a (142 mg, 0.0780 mmol) in trifluoroacetic acid (4.5 mL), water (0.25 mL) and triisopropylsilane (0.25 mL) was stirred for 6 hours. The solution was then concentrated to yield the deprotected hydrazide as a green solid (complete deprotection was confirmed by HRMS). The green solid was then redissolved in water (2 mL) and acetonitrile (2 mL) and benzaldehyde (2 drops) was added, the mixture was then stirred for 30 minutes. The precipitate was isolated and washed with water and acetonitrile to yield the product as a dark purple solid (30 mg, 0.019 mmol, 25 %); 1H NMR (500 MHz, DMSO-<d6>) δ ppm 1.67 (br. s, 4 H, H8), 3.94 (br. s, 8 H, H7), 4.76 (br. s, 4 H, NH), 5.63 (br. s, 4 H, H6), 7.51 (m, 8 H,
H4), 7.63 (m, 8 H, H12), 7.75 (m, 4 H, H11), 7.95 (br. s, 4 H, H11'), 8.12 (br. s, 4 H, H2), 8.39 (br. s, 8 H, H3), 8.84 (m, 2 H, H13), 8.91 (br. s, 10 H, H12, H13'), 11.56 (br. s, 4 H, H10), 11.73 (br. s, 4 H, H10') (cis and trans hydrazone isomers observed in 1:1 ratio); IR (solid state, cm\(^{-1}\)) 3212 (N-H), 1633 (C=O amide), 1608 (C=N); ESI-MS m/z found 1547.5750 [M+H]\(^+\), [C\(_{88}\)H\(_{75}\)N\(_{16}\)O\(_{12}\)]\(^+\) requires 1547.5340

_Tert-butyl (3S)-3-{{4- (7,12-bis (4-{{(1R)-3- (tert-butoxy)-1- {N' -[[tert-butoxy]carbonyl] hydrazinecarbonyl]-3-oxopropyl] carbamoyl}phenyl}-17-{{1S} -3- (tert-butoxy) -1-{N'{{tert-butoxy} carbonyl] hydrazinecarbonyl] -3-oxopropyl] carbamoyl} phenyl} -21,22,23,24- tetraazapentacyclo [16.2.1.1\(^3,6\).1\(^8,11\).1\(^3,16\)]tetracosa-1,3(24),4,6,8,10, 12,14,16(22),17,19-undecaen-2-yl] phenyl) formamido)- 3- {N'{{tert-butoxy}carbonyl] hydrazinecarbonyl} propanoate, 78b

Tetracarboxyphenyl porphyrin (43 mg, 0.055 mmol), PyBOP (172 mg, 0.330 mmol), (S)-tert-butyl 2-(2-amino-4-tert-butoxy-4-oxobutanoyl)hydrazinecarboxylate, 62b (100 mg, 0.330 mmol) and anhydrous diisopropylethylamine (0.11 mL, 0.66 mmol) in anhydrous dimethylformamide (5 mL) were stirred under a nitrogen atmosphere for 18 hours. The reaction mixture was then diluted with ethyl acetate (50 mL) and washed with saturated sodium hydrogen carbonate solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (3 \(\times\) 50 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a purple solid. This was purified by flash column chromatography (1:1 ethyl acetate:dichloromethane then ethyl acetate) to yield the product as a purple solid (103 mg, 0.0533 mmol, 97%); 4H NMR (500 MHz, MeOD) \(\delta\) ppm 1.53 (s, 36 H, H8/H9), 1.55 (s, 36 H, H8/H9), 2.95 (dd, \(J = 17.4, 8.0\) Hz, 4 H, H7), 3.10 (dd, \(J = 17.4, 4.8\) Hz, 4 H, H7'), 5.30 (br. s, 4 H, H6), 5.45 (d, \(J = 1.4\) Hz, 4 H, NH hydrazone), 8.21
(br. s, 8 H, H2), 8.27 (d, J = 6.7 Hz, 8 H, H3), 8.73 (br. s, 8 H, H5), 9.02 (d, J = 6.7 Hz, 8 H, H4); 
$^{13}$C NMR (101 MHz, MeOD + 10% CDCl3) δ ppm 27.4, 27.6, 37.2, 49.3, 80.9, 81.5, 119.3, 
125.9, 133.2, 134.4, 145.4, 149.5, 156.3, 168.4, 170.2, 171.3, 185.1; IR (solid state, cm$^{-1}$) 
3275 (N-H), 1723 (C=O ester), 1711 (C=O carbamate), 1647 (C=O amide); ESI-MS m/z 
found 1931.9891 [M+H]$^+$, [C$_{100}$H$_{123}$N$_{16}$O$_{24}$]$^+$ requires 1931.8896

3(S)-3-{{4-[7,12-bis-(4-[[{(1R)-2-carboxy-1-\{N'-[{(1E)-phenylmethyldiene] hydrazinecarbonyl} ethyl carbamoyl} phenyl]-17-(4-{{[(1S)-2-carboxy-1-\{N'-[{(1E)-phenylmethyldiene] hydrazinecarbonyl}ethyl carbamoyl}phenyl]-21,22,23,24-tetraazapentacyclo[16.2.1.1$^{3,6,11,13,16}$]tetraconta-1,3(24),4,6,8,10,12,14,16(22),17,19-undecaen-2-yl]phenyl}formamido}]-3-\{N'-[{(1E)-phenylmethyldiene] hydrazinecarbonyl}propanoic acid, 79b

$^{Tert}$-butyl (3S)-3-{{4-[7,12-bis(4-{{[(1R)-3-(\{tert-butoxy)carbonyl]hydrazinecarbonyl}-3-oxopropyl]carbamoyl}phenyl]-17-{{[(1S)-3-(\{tert-butoxy)carbonyl]hydrazinecarbonyl]-3-oxopropyl]carbamoyl}phenyl]-21,22,23,24-tetraazapentacyclo[16.2.1.1$^{3,6,11,13,16}$]tetraconta-1,3(24),4,6,8,10,12,14,16(22),17,19-undecaen-2-yl]phenyl}formamido}]-3-\{N'-[(\{tert-butoxy]carbonyl]hydrazinecarbonyl}propanoate (50 mg, 0.026 mmol) in trifluoroacetic acid (4.5 mL), water (0.25 mL) and triisopropylsilane (0.25 mL) was stirred for 6 hours. The solution was then concentrated to yield deprotected hydrazide as a green solid (complete deprotection was confirmed by HRMS). The green solid was then redissolved in water (2 mL) and acetonitrile (2 mL) and benzaldehyde (2 drops) added, the mixture was then stirred for 30 minutes. The precipitate was isolated and washed with water and acetonitrile to yield the product as a dark purple solid (16 mg, 0.0096 mmol, 37%); 4H NMR (500 MHz, DMSO-d$_6$) δ ppm -2.98 -
-2.85 (br. s, 2 H, H1), 2.78 - 3.06 (m, 8 H, H7 + H7'), 5.06 (m, 4 H, H6), 5.87 (m, 1 H, NH), 7.46 (d, J = 6.7 Hz, 8 H, H3), 7.73 (d, J = 6.8 Hz, 4 H, H11), 7.76 (d, J = 6.8 Hz, 4 H, H11'), 8.06 (m, 4 H, H2), 8.34 (m, 16 H, H2, H4), 8.85 (br. s, 8 H, H12 + H12'), 9.08 (m, 2 H, H13), 9.14 - 9.23 (m, 2 H, H13'), 11.54 (br. s, 2 H, H10), 11.67 (br. s, 2 H, H10'), 12.46 (br. s, 4 H, OH) (cis and trans isomers of hydrazone present in 1:1 ratio); IR (solid state, cm⁻¹) 3270 (N-H), 1714 (C=O acid), 1643 (C=O amide), 1607 (C=N); ESI-MS m/z found 1659.5058 [M+H]⁺, [C₉₂H₇₅N₁₆O₁₆]⁺ requires 1659.5547

*Tert*-butyl (4S) -4- {4- [7,12- bis (4- [[(1R)-4- (tert-butoxy) carbonyl] hydrazinecarbonyl]-4-oxobutyl] carbamoyl] phenyl} -17-4-{{{(1S)-4- (tert-butoxy) -1- {N'-[(tert-butoxy) carbonyl] hydrazinecarbonyl] -4- oxobutyl] carbamoyl] phenyl} -21,22,23,24- tetraazapentacyclo [16.2.1.3,6.1⁸,1¹¹.1¹³,1⁶] tetracosa-1,3(24),4,6,8,10,12,14,16(22), 17,19-undecaen-2-yl] phenyl] formamido}-4-{N'-(tert-butoxy)carbonyl] hydrazine carbonyl] butanoate, 78c

Tetracarboxy phenyl porphyrin (42 mg, 0.053 mmol), PyBOP (164 mg, 0.315 mmol), (S)-*tert*-butyl 2-(2-amino-5-*tert*-butoxy-5-oxopentanoyl)hydrazinecarboxylate, 62c (100 mg, 0.315 mmol) and diisopropylethylamine (0.11 mL, 0.63 mmol) in anhydrous dimethylformamide (5 mL) were stirred under nitrogen for 18 hours. The reaction mixture was then diluted with ethyl acetate (50 mL) and washed with saturated sodium hydrogen carbonate solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (3 x 50 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a purple solid. This was purified by flash column chromatography (1:1 ethyl acetate:dichloromethane then ethyl acetate) to yield the product as a purple solid (65 mg, 0.033 mmol, 62 %); ¹H NMR (500 MHz, MeOD) δ ppm 1.51 (s, 36 H, H7/H10), 1.54 (s, 36 H, H7/H10), 2.34 (m, 8 H, H8), 2.63 (m, 8 H, H9), 3.35 (br. s, 4 H, NH hyd), 3.39 (s, 4 H, NH
hyd), 4.92 (br. s, 4 H, H6), 8.00 (br. s, 8 H, H3), 8.18 (br. s, 8 H, H4), 8.61 (m, 8 H, H2); \^13C NMR (101 MHz, MeOD + 10% CDCl\textsubscript{3}) \(\delta\) ppm 27.1, 27.5, 27.7, 31.7, 51.9, 81.0, 119.4, 125.9, 133.2, 134.5, 145.4, 149.5, 156.2, 167.5, 168.4, 170.3, 200.0; IR (solid state, cm\textsuperscript{-1}) 3275 (N-H), 1723 (C=O ester), 1643 (C=O carbamate), 1608 (C=O amide); ESI-MS \(m/z\) found 1988.0341 [M+H]+, [C\textsubscript{104}H\textsubscript{131}N\textsubscript{16}O\textsubscript{24}] requires 1987.9522

\((4S)-4-\left(7,12\text{-bis}\left(4-\left((1R)-3\text{-carboxy}-1-\left(N'\text{-}[\text{(1E)}\text{-phenylmethylidene]}\text{hydrazine carbonyl}\text{propyl}][\text{carbamoyl}][\text{phenyl]}\text{-17}-\left((1S)-3\text{-carboxy}-1-\left(N'\text{-}[\text{(1E)}\text{-phenylmethyldene]}\text{hydrazinecarbonyl}][\text{propyl}][\text{carbamoyl}][\text{phenyl]}\text{-21,22,23,24-tetraaza pentacyclo[16.2.1.1\text{³,}\text{⁶.1\text{⁸,}\text{¹¹.1\text{¹³,}\text{¹⁶}]}\text{tetracosa-1,3(24),4,6,8,10,12,14,16(22),17,19-undecaen-2-yl]}\text{ phenyl}][\text{formamido}]-4-\left(N'\text{-}[\text{(1E)}\text{-phenylmethylidene]}\text{hydrazinecarbonyl}][\text{butanoic acid, 79c}}

\begin{align*}
\text{Tert-butyl (4S) -4-\left(7, 12\text{-bis \(4-\left((1R)-4-\left(\text{tert- butoxy} \right]{\text{carbonyl]}\text{hydrazinecarbonyl\text{-4-oxobutyl} carbamoyl} \text{phenyl]}\text{-17- (4- \left((1S)-4-\left(\text{tert-butoxy)}\text{carbonyl}][\text{hydrazinecarbonyl]}\text{-4-oxobutyl}][\text{carbamoyl}][\text{phenyl]}\text{-21,22,23,24-tetraazapentacyclo[16.2.1.1\text{³,}\text{⁶.1\text{⁸,}\text{¹¹.1\text{¹³,}\text{¹⁶}]}\text{tetracosa-1,3(24),4,6,8,10,12,14,16(22),17,19-undecaen-2-yl]}\text{ phenyl}][\text{formamido}]-4-\left(N'\text{-}[\text{(tert-butoxy)carbonyl}][\text{hydrazine carbonyl]}\text{butanoate, 78b}}
\end{align*}

\(30 \text{ mg, 0.015 mmol}\) in trifluoroacetic acid (4.5 mL), water (0.25 mL) and triisopropylsilane (0.25 mL) was stirred for 6 hours. The solution was then concentrated to yield the deprotected hydrazide porphyrin as a green solid (complete deprotection was confirmed by HRMS). The green solid was then redissolved in water (2 mL) and acetonitrile (2 mL) and benzaldehyde (2 drops) was added, the mixture was then stirred for 30 minutes. The precipitate was isolated and washed with water and acetonitrile to yield the product as a dark purple solid (25 mg, 0.0093 mmol, 62%); \(^1\text{H}

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NMR (500 MHz, DMSO-d$_6$) δ ppm -2.94 (br. s, 2 H, H1), 0.79 (m, 4 H, H7), 1.36 (m, 4 H, H7'), 2.07 (m, 4 H, H8), 4.59 (br. s, 4 H, H6), 5.48 (br. s, 4 H, NH), 7.45 (br. s, 8 H, H3), 7.56 - 7.67 (m, 4 H, H12), 7.73 (m, 4 H, H12'), 7.82 (m, 4 H, H11), 7.87 - 7.98 (m, 4 H, H11'), 8.09 (br. s, 4 H, H2), 8.36 (br. s, 8 H, H4), 8.83 (br. s, 8 H, H3), 8.94 (m, 2 H, H13), 9.04 (br. s, 2 H, H13'), 11.52 (m, 2 H, H10), 11.68 (m, 2 H, H10'), 12.33 (br. s, 4 H, OH) (cis and trans isomers of hydrazone observed in 1:1 ratio); IR (solid state, cm$^{-1}$) 3283 (N-H), 1633 (C=O acid), 1607 (C=O amide), 1529 (C=N); ESI-MS m/z found 1715.5801 [M+H]$^+$, [C$_{96}$H$_{83}$N$_{16}$O$_{16}$]+ requires 1715.6173

1-[2-oxo-2-(pyridin-2-yl)ethyl]pyridin-1-ium iodide, 83$^{143}$

Pyridine (11.5 mL, 0.142 mmol), 2-acetyl pyridine (5 mL, 0.044 mmol) and iodine (3.39 g, 0.0133 mmol) were stirred at 80 °C for 4 hours. The solution was then filtered and the precipitate washed with pyridine. The grey precipitate was then boiled in ethanol with activated charcoal and hot filtered. The filtrate was concentrated to yield the product as a golden solid (6.56 g, 46 %); $^1$H NMR (500 MHz, CDCl$_3$) δ ppm 7.03 (s, 2 H, H5), 7.64 - 7.69 (m, 1 H, H4), 7.97 (td, $J = 7.9, 1.6$ Hz, 1 H, H3), 8.12 (d, $J = 7.9$ Hz, 1 H, H1), 8.19 (dd, $J = 5.9, 4.8$ Hz, 2 H, H7), 8.61 (t, $J = 7.9$ Hz, 1 H, H2), 8.82 (t, $J = 4.8$ Hz, 1 H, H8), 9.26 (d, $J = 5.9$ Hz, 2 H, H6); $^{13}$C NMR (126 MHz, DMSO-d$_6$) δ ppm 48.6, 66.3, 122.0, 127.7, 129.1, 138.1, 146.3, 149.5, 150.4, 191.4; IR (solid state, cm$^{-1}$) 1709 (C=O); ESI-MS m/z found 199.0899 [M]$^+$, [C$_{12}$H$_{11}$N$_2$O]$^+$ requires 199.0871

5-methyl-2-(pyridin-2-yl)pyridine, 85$^{143}$

1-[2-oxo-2-(pyridin-2-yl)ethyl]pyridin-1-ium iodide, 83 (5.00 g, 15.3 mmol), methacrolein (1.26 mL, 15.3 mmol) and ammonium acetate (4.72 g, 61.2 mmol) in methanol (50 mL) were stirred at 65 °C for 16 hours. The solution was diluted with dichloromethane (50 mL) and washed with water; the aqueous phase was extracted with dichloromethane (2 × 50 mL). The combined organic phases were washed with brine (50
mL), dried (sodium sulfate) and concentrated to yield the crude product as a brown oil. This was purified by flash column chromatography (3:7 ethyl acetate:dichloromethane) to yield the product as a pale yellow oil (1.80 g, 10.6 mmol, 69 %); 1H NMR (500 MHz, CDCl₃) δ ppm 2.37 (s, 3 H, H8), 7.27 (ddd, J = 7.5, 4.7, 1.2 Hz, 1 H, H2), 7.61 (dd, J = 7.5, 1.2 Hz, 1 H, H4), 7.78 (td, J = 7.5, 1.7 Hz, 1 H, H3), 8.27 (d, J = 8.0 Hz, 1 H, H5), 8.34 (d, J = 8.0 Hz, 1 H, H6), 8.50 (s, 1 H, H7), 8.66 (d, J = 4.7 Hz, 1 H, H1); 13C NMR (126 MHz, CDCl₃) δ ppm 21.2, 121.4, 122.1, 123.7, 124.8, 127.1, 148.4, 148.9, 149.1, 155.8, 156.2; IR (solid state, cm⁻¹) 1589 (aromatic C=C), 1575 (aromatic C=C), 1558 (aromatic C=C); ESI-HRMS found m/z 171.0920 [M+H]+, [C₁₁H₁₁N₂]⁺ requires 171.0922

6-(Pyridin-2-yl)pyridine-3-carboxylic acid, 69

5-Methyl-2-(pyridin-2-yl)pyridine, 85 (750 mg, 4.41 mmol) and potassium permanganate (2.79 g, 17.6 mmol) in water were heated under reflux for 16 hours. The solution was then cooled to room temperature and filtered through celite. The filtrate was made basic by addition of 1 M sodium hydroxide solution, and washed with dichloromethane (100 mL). The aqueous phase was acidified by addition of acetic acid, and extracted with dichloromethane (5 × 100 mL). The combined organic phases were dried (sodium sulfate) and concentrated, to yield the product as a pale pink solid (547 mg, 3.21 mmol, 73 %); 1H NMR (500 MHz, MeOD) δ ppm 7.51 (ddd, J = 7.8, 5.0, 0.9 Hz, 1 H, H2), 8.00 (td, J = 7.8, 1.7 Hz, 1 H, H3), 8.43 - 8.51 (m, 3 H, H1+H4+H5), 8.72 (d, J = 4.4 Hz, 1 H, H6), 9.25 (s, 1 H, H7); 13C NMR (126 MHz, DMSO-d₆) δ ppm 120.2, 121.2, 124.9, 127.1, 137.5, 138.1, 149.5, 150.1, 154.3, 158.2, 166.2; IR (solid state, cm⁻¹) 2544 (O-H), 1680 (C=O acid); ESI-HRMS found m/z 201.0661 [M+H]+, [C₁₁H₈N₂O₂]⁺ requires 201.0664

Methyl 6-(pyridin-2-yl)pyridine-3-carboxylate, 86

6-(Pyridin-2-yl)pyridine-3-carboxylic acid, 86 (500 mg, 2.50 mmol), anhydrous methanol (30 mL) and thionyl chloride (2.50 mL) were heated under reflux for 16 hours
under a nitrogen atmosphere. The reaction mixture was then cooled to room temperature and quenched with saturated sodium hydrogen carbonate solution (100 mL). The mixture was extracted with dichloromethane (100 mL) and the organic phase washed with 1 M hydrochloric acid (50 mL) and brine (50 mL), dried (sodium sulfate) and concentrated to yield the product as a pale pink solid (425 mg, 1.99 mmol, 79 %); 1H NMR (500 MHz, CDCl₃) δ ppm 3.98 (s, 3H, H8), 7.40 (dd, J = 7.4, 1.4 Hz, 1H, H2), 7.89 (td, J = 7.4, 1.4 Hz, 1H, H3), 8.44 (dd, J = 8.3, 2.0 Hz, 1H, H6), 8.52 (d, J = 7.4 Hz, 1H, H4), 8.55 (d, J = 8.3 Hz, 1H, H5), 8.75 (br. s, 1H, H1), 9.30 (br. s, 1H, H7); 13C NMR (126 MHz, CDCl₃) δ ppm 52.4, 117.2, 120.6, 124.5, 125.7, 137.1, 138.1, 149.4, 150.5, 155.1, 159.5, 165.9; IR (solid state, cm⁻¹) 1716 (C=O ester); ESI-HRMS found m/z 215.0821 [M+H]+, [C₁₂H₁₀N₂O₂]⁺ requires 215.0821

Tris (methyl 6-(pyridin-2-yl) pyridine-3-carboxylate) ruthenium(II) dihexafluorophosphate, 87

Methyl 6-(pyridin-2-yl)pyridine-3-carboxylate, 86 (250 mg, 1.17 mmol), Ru(II)(DMSO)₄Cl₂ (177 mg, 0.364 mmol), and silver nitrate (171 mg, 0.728 mmol) in ethanol (20 mL) were heated under reflux for 6 days. The reaction mixture was then filtered through celite, and the celite pad washed thoroughly with dichloromethane. The filtrate was then concentrated and the resulting red solid redissolved in water. An excess of ammonium hexafluorophosphate was added and the red precipitate isolated to yield the product as a red solid (308 mg, 0.298 mmol, 82 %); 1H NMR (500 MHz, DMSO-d₆) δ ppm 3.18 (s, 4.5 H, H8), 3.80 (s, 4.5 H, H8), 7.60 (m, 3 H, H2), 7.82 (m, 3 H, H3), 7.05 (m, 3 H, H6), 8.24 (m, 3 H, H4), 8.55 (m, 3 H, H5), 8.95 (m, 6 H, H1+H7) (fac and mer isomers seen on Me in 1:1 ratio); IR (solid state, cm⁻¹) 1717 (C=O ester); ESI-MS m/z found 372.0635 [M]²⁺, [C₃₆H₃₈N₄O₆Ru]²⁺ requires 372.0635

Tris (6-(pyridin-2-yl)pyridine-3-carboxylic acid) ruthenium(II) dichloride, 88
Tris (methyl 6- (pyridin-2-yl) pyridine-3-carboxylate) ruthenium(II) dihexafluorophosphate, 87 (400 mg, 0.387 mmol) was stirred in 1 M sodium hydroxide solution and ethanol for 2 hours. The reaction mixture was then concentrated and redissolved in water. The resulting solution was neutralized by addition of 1 M hydrochloric acid and concentrated. The salt was removed by dissolving the red solid in repeatedly in a minimal amount of methanol and filtering the precipitated salt, until no salt was visible, to yield the product as a red solid (298 mg, 0.387 mmol, quant.); 1H NMR (500 MHz, D2O) δ ppm 7.24 - 7.41 (m, 3 H, H2), 7.73 - 7.83 (m, 3 H, H3), 7.88 (s, 3 H, H6), 7.95 - 8.06 (m, 3 H, H4), 8.17 (br. s, 3 H, H5), 8.25 - 8.39 (m, 3 H, H1), 8.48 - 8.57 (m, 3 H, H7); IR (solid state, cm−1) 3392 (O−H), 1614 (C=O acid); ESI-MS m/z found 351.0410 [M]+; [C33H24N6O6Ru]+ requires 351.0400

N-[(1S)-2-(tert-butoxy)-1-{N’-[2,2’-bipyridine]-5-carboxamide, 70

6-(pyridin-2-yl)pyridine-3-carboxylic acid (200 mg, 1.00 mmol) in thionyl chloride (10 mL) was heated under reflux for 16 hours. The solvent was then removed in vacuo and the acid chloride flushed with nitrogen and used immediately. The acid chloride was redissolved in anhydrous chloroform (20 mL) under a nitrogen atmosphere and tert-butyl 2-(2-amino-3-tert-butoxypropanoyl)hydrazinecarboxylate, 62a (303 mg, 1.10 mmol) and anhydrous disopropylethylamine (0.19 mL, 1.10 mmol) were added. The reaction mixture was then heated under reflux for 16 hours. The reaction mixture was then washed with saturated sodium hydrogen carbonate solution (50 mL), 1 M hydrochloric acid (50 mL) and brine (50 mL). The organic phase was dried (sodium sulfate) and concentrated to yield the crude product as a pale pink solid. This was purified by flash column chromatography (10 % methanol in dichloromethane) to yield the product as a pink solid (30 mg, 0.066 mmol, 7 %); 1H NMR (500 MHz, CDCl3) δ ppm 1.30 (s, 9 H, H11/H14), 1.50 (s, 9 H, H11/H14), 2.06 (m, 1 H, H10), 2.36 (t, J = 8.5 Hz, 1 H, H10′), 4.00 (dd, J = 8.5, 4.5 Hz, 1 H, H9), 4.72 (br. s, 1 H, NH), 6.62 (br. s, 1 H, NH), 7.43 (m, 2 H, H5 + H6), 7.93 (t, J = 6.9 Hz, 1 H, H4), 8.29 (d, J = 8.2 Hz, 1 H, H1), 8.51 (dd, J = 14.5, 8.2 Hz, 1 H, H2), 8.77 (dd, J =
14.5, 6.9 Hz, 1 H, H3), 9.16 (s, 1 H, H7); IR (solid state, cm\(^{-1}\)) 3273 (N-H), 1699 (C=O amide), 1651 (C=O amide); ESI-MS \(m/z\) found 458.2404 [M+H]\(^+\), \([C_{23}H_{32}N_3O_5]\)^+ requires 458.2403

**4-Methyl-2-(pyridin-2-yl)pyridine, 89**

![Structure of 4-Methyl-2-(pyridin-2-yl)pyridine](image)

1-[2-Oxo-2-(pyridin-2-yl)ethyl]pyridin-1-ium iodide, **83** (4.04 g, 12.4 mmol), crotonaldehyde (0.97 mL, 12.4 mmol) and ammonium acetate (3.82 g, 49.6 mmol) in methanol (50 mL) were stirred at 65 °C for 16 hours. The solution was concentrated and the orange solid diluted with petroleum ether (100 mL) and washed with water (100 mL), and the aqueous phase extracted with petroleum ether (2 \times 50 mL). The combined organic phases were washed with brine (50 mL), dried (sodium sulfate) and concentrated to yield the crude product as a yellow solid (501 mg, 2.95 mmol, 24 %), the crude product was used without further purification; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 2.35 (s, 3 H, H8), 7.04 (d, \(J = 4.7\) Hz, 1 H, H6), 7.18 - 7.24 (m, 1 H, H2), 7.72 (td, \(J = 7.9, 1.8\) Hz, 1 H, H3), 8.15 (s, 1 H, H5), 8.31 (d, \(J = 7.9\) Hz, 1 H, H4), 8.45 (d, \(J = 4.9\) Hz, 1 H, H1), 8.59 (d, \(J = 4.7\) Hz, 1 H, H7); \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) ppm 21.2, 121.3, 121.9, 123.7, 124.7, 137.0, 148.1, 149.1, 149.1, 156.0, 156.4; IR (solid state, cm\(^{-1}\)) 1744 (aromatic C=C), 1602 (aromatic C=C), 1582 (aromatic C=C); ESI-HRMS found \(m/z\) 193.0789 [M+Na]\(^+\), \([C_{11}H_{10}N_2Na]\)^+ requires 193.0742

**2-(Pyridin-2-yl)pyridine-4-carboxylic acid, 90**

![Structure of 2-(Pyridin-2-yl)pyridine-4-carboxylic acid](image)

5-Methyl-2-(pyridin-2-yl)pyridine, **89** (450 mg, 2.64 mmol) and potassium permanganate (1.67 g, 10.6 mmol) in water (50 mL) were heated under reflux for 16 hours. The solution was then cooled to room temperature, filtered through celite and the filtrate concentrated, to yield the product as a white solid (415 mg, 2.07 mmol, 79 %); \(^1\)H NMR (500 MHz, MeOD) \(\delta\) ppm 7.38 (t, \(J = 6.9\) Hz, 1 H, H2), 7.84 (d, \(J = 5.0\) Hz, 1 H, H6), 7.87 (t, \(J = 6.9\) Hz, 2 H, H3), 8.30 (d, \(J = 6.9\) Hz, 2 H, H4), 8.59 (d, \(J = 6.9\) Hz, 1 H, H1), 8.73 (d, \(J = 5.0\) Hz, 1 H, H7), 8.76 (s, 1 H, H5); \(^{13}\)C NMR (126 MHz, D\(_2\)O) \(\delta\) ppm 120.7, 122.0, 123.2,
124.5, 138.2, 145.9, 148.7, 154.2, 155.2, 162.3, 172.5; IR (solid state, cm\(^{-1}\)) 2460 (O-H), 1709 (C=O acid); ESI-HRMS found \(m/z\) 200.0660 [M]\(^+\), \([\text{C}_{11}\text{H}_{9}\text{N}_{2}\text{O}_{2}]^+\) requires 200.0586

**Methyl 2-(pyridin-2-yl)pyridine-4-carboxylate, 91**

![Methyl 2-(pyridin-2-yl)pyridine-4-carboxylate](image)

6-(Pyridin-2-yl)pyridine-3-carboxylic acid, 90 (2.20g, contaminated with salts), methanol (100 mL) and thionyl chloride (15 mL) were heated under reflux for 16 hours. The reaction mixture was then cooled to room temperature and quenched with saturated sodium hydrogen carbonate solution (100 mL) and extracted with dichloromethane (100 mL). The organic phase was washed with 1 M hydrochloric acid (50 mL) and brine (50 mL), dried (sodium sulfate) and concentrated. The resulting beige solid was redissolved in a minimum amount of methanol, and precipitated with water. The resulting solution was filtered and the precipitate washed with water, to yield the product as a beige solid (310 mg, 1.45 mmol); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) ppm 4.00 (s, 3 H, H8), 7.28 (dd, \(J = 7.7, 4.5\) Hz, 1 H, H2), 7.74 - 7.83 (m, 2 H, H7 and H3), 8.35 (d, \(J = 7.7\) Hz, 1 H, H4), 8.65 (d, \(J = 4.5\) Hz, 1 H, H1), 8.76 (d, \(J = 5.0\) Hz, 1 H, H6), 8.87 (s, 1 H, H5); \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) ppm 52.7, 120.4, 121.3, 122.8, 124.2, 137.0, 138.5, 149.4, 150.0, 155.4, 157.4, 165.8; IR (solid state, cm\(^{-1}\)) 1720 (C=O ester); ESI-HRMS found \(m/z\) 215.0817 [M+H]\(^+\), \([\text{C}_{12}\text{H}_{10}\text{N}_{2}\text{O}_{2}]^+\) requires 215.0821

**Tris (methyl 2- (pyridin-2-yl) pyridine -4- carboxylate) ruthenium(II) dihexafluorophosphate, 92**

![Tris (methyl 2- (pyridin-2-yl) pyridine -4- carboxylate) ruthenium(II) dihexafluorophosphate](image)

Methyl 2-(pyridin-2-yl)pyridine-4-carboxylate, 91 (200 mg, 0.934 mmol), Ru(II)(DMSO)\(_4\)Cl\(_2\) (141 mg, 0.292 mmol), silver nitrate (99 mg, 0.58 mmol) and ethanol (25 mL) were heated under reflux for 7 days. The resulting mixture was cooled and filtered through celite. The celite pad was then washed with dichloromethane until all of the red compound had washed through. The filtrate was then concentrated and the resulting red solid redissolved in water. An excess of ammonium hexafluorophosphate
was added, and a red precipitate formed. The mixture was filtered to yield the product as a red solid (256 mg, 0.248 mmol, 85 %); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ ppm 3.90 (s, 9 H, H8), 7.52 (s, 3 H, H5), 7.92 (m, 6 H, H7+H3), 8.00 (d, $J = 6.9$ Hz, 3 H, H4), 8.15 (d, $J = 17.1$ Hz, 3 H, H1), 8.35 (m, 3 H, H2), 8.75 (dd, $J = 17.1, 8.5$ Hz, 3 H, H3); IR (solid state, cm$^{-1}$) 1721 (C=O ester); ESI-MS m/z found 372.0636 [M$^{2+}$], [C$_{36}$H$_{30}$N$_6$O$_6$]$^{2+}$ requires 372.0635

\[ \text{Tris (2-(pyridin-2-yl)pyridine-4-carboxylic acid) ruthenium dichloride, 64} \]

\[ \text{Ru(bpy)}_3^{2+} \]

\[ \text{Tris (methyl 2-(pyridin-2-yl)pyridine-4-carboxylate) ruthenium(II) dihexafluorophosphate (100 mg, 0.0977 mmol), in ethanol (2.5 mL) and 10\% sodium hydroxide solution (2.5 mL) were stirred for 2 hours. The resulting solution was neutralized with 1 M hydrochloric acid and concentrated. The resulting solid was dissolved in methanol and filtered. The red filtrate was concentrated and the process repeated, until no more salt was visible. This gave the product as a red solid (56 mg, 0.072 mmol, 74 \%).} \]

$^1$H NMR (500 MHz, D$_2$O) $\delta$ ppm 7.37 (t, $J = 5.7$ Hz, 3 H, H1), 7.65 (d, $J = 4.2$ Hz, 3 H, H6), 7.72 - 7.80 (m, 3 H, H4), 7.87 (td, $J = 5.7, 3.3$ Hz, 3 H, H2), 8.05 (dt, $J = 8.5, 5.7$ Hz, 3 H, H3), 8.57 (d, $J = 7.7$ Hz, 3 H, H7), 8.80 (s, 3 H, H5); IR (solid state, cm$^{-1}$) 3223 (O-H), 1663 (C=O acid) ESI-MS m/z found 351.0376 [M$^{2+}$], [C$_{33}$H$_{24}$N$_6$O$_6$Ru]$^{2+}$ requires 351.0400

6.2 Biophysical analyses- General considerations chapter 2

All stocks for luminescence intensity assays were made up in 5 mM sodium phosphate, pH 7.5 buffer. Ru(II)(bpy)$_3$ complex stocks were made up to 2 mM. Horse heart and yeast cyt c was obtained from Sigma Aldrich, and used without further purification. Cyt c stocks were made up to ~1 mM, and the concentration accurately determined using the molar extinction coefficient at 550 nm of $2.95 \times 10^4$ mol$^{-1}$ dm$^3$ cm$^{-1}$ for horse heart cyt c$^{202}$ and $2.11 \times 10^4$ mol$^{-1}$ dm$^3$ cm$^{-1}$ for yeast cyt c$^{202}$ after reduction by addition of one microspatula of sodium dithionite. Assays with chemically oxidized cyt c in ascorbate containing buffer used cyt c oxidized with K$_3$Fe(CN)$_6$ followed by dialysis into 5 mM sodium phosphate, 2 mM sodium ascorbate, pH 7.5 buffer, to remove the excess K$_3$Fe(CN)$_6$. The concentration of oxidized cyt c was determined by using the molar extinction coefficient at 410 nm of $1.061 \times 10^5$ mol$^{-1}$ dm$^3$ cm$^{-1}$. All other buffers used were at 5 mM concentration, 0.2 mg mL$^{-1}$ BSA, pH 7.5, unless otherwise stated.

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6.2.1 Luminescence quenching assays (Chapter 2)

In all assays the Ru(II)(bpy)$_3$ complex concentration was kept constant, with the concentration of cyt $c$ being varied through the assay, as described below. Results obtained were fitted, using OriginPro 9, to a 1:1 binding isotherm (Eq. 6.1).

$$I = \frac{m[(a+b+K)-(a+b+K)^2-4ab]}{2a}$$

Eq. 6.1

Where $I =$ change in relative luminescence intensity ($I/I_0$), $m =$ maximum value of $I$, $a =$ concentration of Ru(II)(bpy)$_3$ complex, $K =$ dissociation constant, $b =$ concentration of protein added

6.2.1.1 Fluorometer

Assays were measured on a Jobin-Yvon Spex Fluorolog-3 fluorometer. Measurements were taken in a 4 mL quartz cuvette with excitation at 467 nm and emission measured over the range 575 – 675 nm, with 10 nm slit widths on both excitation and emission. Measurements were taken on triplicate titrations.

1. 2.5 mL of the Ru(II)(bpy)$_3$ complex at an appropriate concentration was put in the cuvette
2. The emission spectrum was taken
3. 10 $\mu$L of a solution with an appropriate cyt $c$ concentration with the same concentration of Ru(II)(bpy)$_3$ complex (so as to keep the complex concentration constant) was added
4. The emission spectrum was taken
5. This was repeated ~15 times
6. The peak maxima were taken, and these values used (as I)

6.2.1.2 Plate reader

Assays were scanned using a Perkin Elmer EnVision™ 2103 MultiLabel plate reader, with excitation at 467 nm, and emission at 630 nm fixed wavelength. A 2/3 dilution regime in a 384 well plate (Optiplate) was used, with each result measured in triplicate:

1. 25 $\mu$L of buffer was added to all wells
2. 50 $\mu$L of cyt $c$ solution was added to the first well of a row
3. Serial dilution, taking 50 $\mu$L from the first well and transferring to the second well mixing, and repeating up to the 23rd well, leaving the last well without any protein
4. 25 μL of Ru(II)(bpy)$_3$ complex solution was added to each well
5. The plate was incubated for 45 minutes before reading (excitation 467 nm, emission 630 nm)

6.2.2 UV/Vis ascorbate reduction

A 500 μL quartz cuvette was charged with 400 μL of a 20 μM solution of cyt c and 20 μM Ru(II)(bpy)$_3$ complex in 5 mM sodium phosphate buffer, pH 7.5. 100 μL of 10 mM ascorbate in 5 mM sodium phosphate, pH 7.5 was added, and the spectrophotometer started immediately. Absorbance at 550 nm was monitored every 0.5 seconds for 5 minutes. The absorbances were normalised (against A$_{550}^{max}$-A$_{550}^{min}$) and plotted against time.

6.2.3 Protein NMR

Sensitivity enhanced $^1$H-$^{15}$N HSQC NMR correlation spectra of ligand-bound and unbound forms of horse heart cyt c, purchased from Sigma Aldrich, were carried out at natural abundance using a 950 MHz Bruker Ascend$^\text{TM}$ Aeon spectrometer operating at a proton ($^1$H) resonance frequency of 950.13 MHz equipped with a Bruker TCI triple-resonance cryo-probe. NMR acquisitions were carried out in 5 mM sodium phosphate, 2 mM sodium ascorbate, pH 7.25 buffer. For cyt c alone, spectra were taken at 2 mM protein concentration, with a total volume of 600 μL. With Ru(II)(bpy)$_3$ complex 31 and 26, 1 mM cyt c and 0.5 mM Ru(II)(bpy)$_3$ complex were used, to a total volume of 600 μL. Spectra were analysed using the CcpNmr Analysis software package and the chemical shift perturbations were calculated as the square root of the sum of the isotope weighted shift differences squared (Eq. 6.2),

$$\Delta\delta = \sqrt{(\Delta\delta_N)^2 + \left(\frac{\gamma_H}{\gamma_N}\right)^2(\Delta\delta_H)^2}$$

Eq. 6.2

Where $\Delta\delta$ is the overall change in chemical shift, $\Delta\delta_N$ is the change in the nitrogen dimension and $\Delta\delta_H$ is the change in the proton dimension, respectively. The change in the proton dimension is scaled by the ratio of the gyromagnetic ratio of $^{15}$N ($\gamma_N$) and $^1$H ($\gamma_H$) to account for the larger chemical shift range of $^{15}$N.

6.3 Hydrazone Exchange Studies (Chapter 3)

All hydrazone exchange reactions were carried out in HPLC vials and were followed using high resolution mass spectrometry, using a Bruker Daltonics micrOTOF Premier Mass Spectrometer, using 10 μL injections and summing the masses over the range 1.0 to 3.0 minutes. The intensity of the maximum peak for each of the successive hydrazone
exchanges was taken and the percentage associated with each of the Ru(II)(bpy)$_3$ or porphyrin peaks calculated.

6.3.1 Ru(II)(bpy)$_3$ Complexes

Ru(II)(bpy)$_3$ hydrazone complex stocks were made up at 2 mM concentration in 1:1 acetonitrile:water. Aniline and aldehyde (2,4-dimethoxy benzaldehyde) stocks were made to 1 M concentration in acetonitrile.

6.3.1.1 Hydrazone exchange reactions

In hydrazone exchange reactions 1 mL of 100 μM Ru(II)(bpy)$_3$ hydrazone complex 46a, 10 mM aldehyde and 10 mM aniline in 1:1 acetonitrile water were incubated in an HPLC vial and mass spectra were obtained at appropriate time periods.

6.3.2 Porphyrins

Hydrazone functionalised porphyrins 79 were made up to 5 mM concentration in DMSO and were stored in plastic Eppendorf tubes. 1 M stocks of catalyst (aniline and anthranillic acid) and aldehydes (2,4-dimethoxy benzaldehyde, 4-carboxy benzaldehyde, 4-methyl ester benzaldehyde) were made up in DMSO. 4-hydroxy benzaldehyde stock were made up to 0.5 M in DMSO.

6.3.2.1 Hydrazone exchange reactions

In all exchange reactions the hydrazone functionalised porphyrin 79 in DMSO was added to a solution of the aldehyde and catalyst to give a total concentration of 10 % DMSO in 5 mM ammonium acetate buffer, pH 6.75, to a final porphyrin concentration of 100 μM and stated concentrations of other components. For time courses, mass spectra were obtained at appropriate time points (usually 0.5, 1, 2, 4, 6, 9, 12 and 24 hours). For measurements at single time points, mass spectra were obtained after 24 hours incubation. For pre-incubated samples, the pre-incubated mixture was left for 24 hours, and a mass spectrum obtained, prior to addition of further components.

6.3.2.2 Protein incubation

A 200 μL solution containing 100 μM benzaldehyde hydrazone porphyrin 79, 2.5 mM of any aldehyde, 10 mM aniline in 10 % DMSO in 5 mM NH$_4$OAc, pH 6.75 buffer was incubated for 24 hours. At which point 20 μL of 1 mM protein stock in 5 mM sodium phosphate, pH 7.5 buffer or 5 mM sodium phosphate, pH 7.5 buffer alone (for no protein comparison) was added, and incubated for a further 24 hours. The reaction was then quenched by addition of 20 μL of NH$_4$OH solution.
6.4 **Biophysical analyses- General considerations chapter 4**

All arrays were performed in 5 mM sodium phosphate, pH 7.5 buffer. All protein stocks, other than Mcl-1 and hDM2, were made up from freeze-dried protein, purchased from major suppliers, into 5 mM sodium phosphate, pH 7.5 at ~ 1 mM concentration. Accurate concentrations were determined by UV/Vis using extinction coefficients (at 280 nm) of 36, 50, 43.8, 57.6 and 9.9 mol⁻¹ dm³ cm⁻¹ for lysozyme, α-chymotrypsin, BSA, papain and ribonuclease A respectively and for cyt c, as for chapter 2.

All arrays were performed in 384 Optiplate well plates and were scanned using a Perkin Elmer EnVision™ 2103 MultiLabel plate reader.

6.4.1 **Ru(II)(bpy)₃ complexes and proteins**

To each well was added 20 μL of 5 μM Ru(II)(bpy)₃ complex solution and 20 μL of 20 μM protein solution or 5 mM sodium phosphate, pH 7.5 buffer. The plate was incubated for 45 minutes before scanning using fixed wavelengths, excitation 467 nm, emission 630 nm, and using monochromators, excitation 467 nm, emission range 500 – 800 nm, 3 nm step, 100 flashes. The peak maxima/ intensities were taken. The values without protein were averaged over the triplicate wells, and the percentage difference for each of the other wells containing the same Ru(II)(bpy)₃ complex calculated. Each of these results was used for statistical analysis.

6.4.2 **Ru(II)(bpy)₃ complexes, FITC-NOXA B (R-A) tracer and proteins**

To each well was added 20 μL of 7.5 μM Ru(II)(bpy)₃ complex solution (or 5 mM sodium phosphate buffer, pH 7.5), 20 μL of 30 μM protein solution (or 5 mM sodium phosphate, pH 7.5 buffer) and 20 μL of 1.5 μM FITC-NOXA-B (R-A) peptide (or 5 mM sodium phosphate, pH 7.5 buffer). On each plate wells without protein were run in quadruplicate. The plate was incubated for 2 and 20 hours prior to scanning using fixed wavelengths, excitation 467 nm, emission 630 nm, and using monochromators, excitation 467 nm, emission range 480 - 750 nm, 3 nm step, 100 flashes. The peak maxima/intensities for both luminescence bands were taken (emission 520 nm and 630 nm). The values obtained for wells with no protein present were averaged over the quadruplicate wells, all other data used was from each well individually. The percentage difference for each well for that with protein to without protein was calculated and used for statistical analysis.
6.4.3 **Linear discriminant analysis**

Microsoft Excel was used to calculate the percentage differences from no protein for each of the individual wells. Linear discriminant analysis was carried out using XLstat software, then plotted using OriginPro 9. Confidence ellipses were obtained using OriginPro 9.4.

6.5 **General considerations for Appendix IV - DCC array studies with hydrazone functionalised Ru(II)(bpy)$_3$ complexes**

The glycine hydrazide Ru(II)(bpy)$_3$ complex 49 was made up to 10 mM concentration in 50 mM ammonium acetate, pH 6.2 buffer. Aniline stocks were made up to 1 M in DMSO, and aldehyde (4-carboxy benzaldehyde, 2,4-dimethoxy benzaldehyde, 4-hydroxy benzaldehyde, 2,4-dihydroxy benzaldehyde, furfural, 4-nitro benzaldehyde, tert-butoxy benzaldehyde, benzaldehyde, 3-pyridine carboxaldehyde, 4-chloro benzaldehyde) stocks were made up to 100 mM in DMSO. Protein stocks were made up to ~1 mM in 5 mM sodium phosphate, pH 7.5 and concentrations measured as for Chapter 4.

DCC arrays were set up in 96 well plates (Optiplate) in triplicate. In each well 250 μL of 50 μM glycine hydrazide complex, 10 mM aniline, 300 μM each aldehyde and 50 μM protein in 10% DMSO in 50 mM ammonium acetate, pH 6.2 buffer was incubated for 18 hours at room temperature. At which point the reaction was quenched by addition of 4 μL of 1 M NaOH. The contents of each well were transferred to a vivaspin 500 protein concentrator MWCO 5 kDa, and centrifuged at 13 000 g for 22 minutes. 4 μL of 1 M NaOH followed by 200 μL of NH$_4$OAc, pH 6.2 buffer was added to the concentrate and the concentrator centrifuged at 8 000 g for 40 minutes. The flow through was analysed by analytical HPLC (Rapid 5-95 Methanol+TFA gradient using an Ascentis Express C18 column) at 280 nm, and the peak integrations for the major peaks taken and used for analysis.

6.6 **General considerations for Appendix V - Stabilisation of the p53 tetramerisation domain**

The two peptides were obtained from ProteoGenix. Peptide stocks were made up to 1 mg mL$^{-1}$ in water, and neutralised to pH 7.0 by addition of 10 mM hydrochloric acid or 10 mM ammonium hydroxide solution, or in 5 mM sodium phosphate, pH 7.5. The accurate peptide concentration was determined by UV/Vis absorption at 280 nm (ε = 1490 M$^{-1}$ cm$^{-1}$ for both peptides). Ru(II)(bpy)$_3$ complex stocks were made up to 1 mM in water, neutralised to pH 7.0 by addition of 10 mM hydrochloric acid or 10 mM ammonium hydroxide solution, or in 5 mM sodium phosphate, pH 7.5 buffer.
6.6.1 Circular dichroism

Circular dichroism was performed on an Applied Photophysics ChiraScan Apparatus using the associated Software. The range 180-260 nm was scanned using point time 1 s, 1 nm per point, step = 1 nm, 5 nm bandwidth and path length 10 mm. All samples were run in either 5 mM sodium phosphate, pH 7.5 buffer or water. 100 μM peptide concentrations were used for all spectra, with 0, 100 or 400 μM Ru(bpy)$_3$ complex concentration.

For temperature ramp experiments, the temperature was ramped in steps of either 1 °C or 2.5 °C over the range 20 – 90 °C, with a temperature equilibration time of 4 minutes between the temperature increase and obtaining the CD spectra. A final spectrum was obtained after the chamber had cooled to 20 °C. The ellipticity at 222 nm (corresponding to helical content) was used to calculate the fraction folded (Eq. 6.3).

$$\alpha = \frac{\theta(T) - \theta_f}{\theta_u - \theta_f}$$

Eq. 6.3

\(\alpha = \) fraction folded, \(\theta(T) = \) ellipticity at temperature \(T\), \(\theta_f = \) \(\theta\) at 20 °C and \(\theta_u = \) \(\theta\) at 90 °C
7 Appendices

7.1 Appendix I- Debye Hückel equation

The Debye-Hückel limiting law derives from the idea that near any ion in solution, counter ions will cluster, and thus lower the chemical potential of that original ion, making it act non-ideally and lowering its electrostatic interactions.\(^{208}\) This will affect interactions within the solution, for example the binding between the Ru(bpy)\(_3\) complex and cyt c, both of which are charged and thus be dependent on factors including the concentration, size and charge of the ions.

In order to see how this can provide information on the binding between the two species, it is first necessary to understand the fundamentals of chemical reaction thermodynamics. Considering the reaction equation for the dissociation of the Ru(II)(bpy)\(_3\)-cyt c interaction-

\[
\text{Ru(bpy)}_3\text{-Cyt c} \rightarrow \text{Cyt c} + \text{Ru(bpy)}_3
\]

Using standard thermodynamic notation, the Gibbs free energy (\(\Delta G\)) can be expressed in terms of the chemical potentials (\(\mu\)) of each of the species

\[
\Delta G = \mu_A + \mu_B - \mu_{A,B} \quad \text{Eq. 7.1}
\]

The chemical potential of a species \(i\) can be expressed in terms of the standard chemical potential (\(\mu^0\)) of that species and the activity (\(a\)), the effective concentration of the species in a mixture

\[
\mu_i = \mu^0_i + RT \ln a_i \quad \text{Eq. 7.2}
\]

The activity can further be defined in terms of the molality (\(m\)) (the concentration of solute in a solvent, in water this is the molarity) and an activity coefficient (\(\gamma\)), which depends on the composition, molality and temperature of the solution

\[
a_i = \gamma_i \frac{m_i}{m_i^0} \quad \text{Eq. 7.3}
\]

Combining Eq. 6.2 and Eq. 6.3

\[
\mu_i = \mu^0_i + RT \ln \frac{m_i}{m_i^0} + RT \ln \gamma_i \quad \text{Eq. 7.4}
\]
If we assume that the molality is the ideal molality \( m_i = m_i^0 \), then

\[
\mu_i = \mu_i^0 + RT \ln \gamma_i
\]

Eq. 7.5

This equation can then be put back into Eq. 6.1

\[
\Delta G = \mu_A^0 + \mu_B^0 - \mu_C^0 + RT \ln \gamma_A + RT \ln \gamma_B - RT \ln \gamma_C
\]

Eq. 7.6

As

\[
\Delta G^0 = \mu_A^0 + \mu_B^0 - \mu_{AB}^0
\]

Eq. 7.7

Then

\[
\Delta G = \Delta G^0 + RT \ln \frac{\gamma_{AB}}{\gamma_{A,B}}
\]

Eq. 7.8

Simplifying

\[
\Delta G = \Delta G^0 + RT \ln \frac{\gamma_{AB}}{\gamma_{A,B}}
\]

Eq. 7.9

Inputting the Gibbs free energy isotherm-

\[
\Delta G = -RT \ln K
\]

Eq. 7.10

Gives

\[
-RT \ln K = -RT \ln K^0 + RT \ln \frac{\gamma_{AB}}{\gamma_{A,B}}
\]

Eq. 7.11

Simplifying this gives

\[
\ln K = \ln K^0 - \ln \frac{\gamma_{AB}}{\gamma_{A,B}}
\]

Eq. 7.12

In an ideal (infinitely dilute) solution or gas, the \( \gamma \) values are 1, thus making

\[
\ln K = \ln K^0
\]

Eq. 7.13

An ionic solution, however is not ideal and \( \gamma \neq 1 \), therefore we need to compute values for \( \gamma \), this is done using the Debye-Hückel approximation,\textsuperscript{208,209} where it is assumed that the activities (a) or activity coefficients (\( \gamma \)) are assumed to be dependent on the ionic strength of the solution (I) and not on the composition of the solution.

The Debye-Hückel approximation-

\[
\log_{10} \gamma = -|z_+ z_-| A(I)^{1/2}
\]

Eq. 7.14

Where, \( z_+ \) and \( z_- \) are the charges on the two species, A is a constant which is empirically derived, and in aqueous solutions, at 25 °C is 0.509.

A second approximation is the Güntelberg approximation which is empirically derived and valid to higher ionic strengths (<100 mM), and assigns some size to the ions (as opposed to the Debye-Hückel equation, which assumes point charges).\textsuperscript{209}

\[
\log_{10} \gamma_i = \frac{A_i k_i}{1 + k_i I}
\]

Eq. 7.15
And

\[ \log_{10} r_{ij} = \frac{Azi_x |\sqrt{T}|}{1 + \sqrt{T}} \]  \hspace{1cm} \text{Eq. 7.16}

Converting to \ln

\[ \ln \gamma_i = \frac{A}{\log_{10} e} \frac{zi_x |\sqrt{T}|}{1 + \sqrt{T}} \]  \hspace{1cm} \text{Eq. 7.17}

\[ \ln \gamma_{ij} = \frac{A}{\log_{10} e} \frac{zi_x |\sqrt{T}|}{1 + \sqrt{T}} \]  \hspace{1cm} \text{Eq. 7.18}

Inputting Eq. and Eq. into Eq. 6.13 for the dissociation reaction

\[ \ln K = \ln K^0 - \frac{A}{\log_{10} e} \frac{zi_x A |\sqrt{T}|}{1 + \sqrt{T}} \]  \hspace{1cm} \text{Eq. 7.19}

Simplifying

\[ \ln K = \ln K^0 - \frac{ART}{\log_{10} e} \left( \frac{zi_x A |\sqrt{T}|}{1 + \sqrt{T}} \right) \]  \hspace{1cm} \text{Eq. 7.20}

Changing from \ln to \log_{10} and simplifying

\[ \log_{10} K = \log_{10} K^0 - A \left( \frac{zi_x A |\sqrt{T}|}{1 + \sqrt{T}} \right) \]  \hspace{1cm} \text{Eq. 7.21}

At 298 K in water \( A = -0.509 \), therefore

\[ \log_{10} K = \log_{10} K^0 - 0.509 \left( \frac{zi_x A |\sqrt{T}|}{1 + \sqrt{T}} \right) \]  \hspace{1cm} \text{Eq. 7.22}
7.2 Appendix II- Monosubstituted Ru(II)(bpy)₃ complex synthesis

The synthesis of monosubstituted Ru(II)(bpy)₃ complexes was attempted for the use in DCC studies as there would be fewer exchangeable groups compared to the disubstituted Ru(II)(bpy)₃ complexes (3 versus 6). This would decrease the number of peaks present in the mass spectrum, and also potentially increase the rate of reaching equilibrium. The use of monosubstituted bipyridine ligands, however, does lead to more stereoisomers of the Ru(II)(bpy)₃ complexes formed, as fac and mer isomers as well as Δ and Λ isomers possible, will be formed.

7.2.1 5’ monosubstituted bipyridine synthesis

The Wilson group have previously reported the use of 5’ monosubstituted Ru(II)(bpy)₃ complexes for binding to cyt c. It was therefore decided to synthesise a 5’ acid complex, via a similar synthetic route (Scheme 7.1). The synthesis uses a Kroehnke method to access the bipyridine by reaction of the Kroehnke reagent with methacrolein to give a 5’ methyl substituted bipyridine. This can be oxidised to the corresponding carboxylic acid with potassium permanganate, followed by methyl ester formation to form, ruthenium(II) complexation to give and subsequent deprotection, to yield the 5’ acid Ru(II)(bpy)₃ complex

![Scheme 7.1 Synthesis of 5’ monosubstituted bipyridine Ru(II)(bpy)₃ complex 65](image)

7.2.2 4’ monosubstituted bipyridine complex synthesis

While the 5’ bipyridine Ru(II)(bpy)₃ complexes have been used in the Wilson group previously, 4’ bipyridines have not been reported by the group. However, the 4’ substituted Ru(II)(bpy)₃ complexes offer advantages over the corresponding 5’ substituted Ru(II)(bpy)₃ complexes, including higher quantum yields of the luminescence, making them much more compatible with luminescence assays and in cellulo visualisation.
Scheme 7.2 4' monosubstituted Ru(II)(bpy)$_3$ complex synthesis using Kroehnke methodology

After various attempts at palladium catalysed cross coupling, using Suzuki and Negishi couplings yielded little of the bipyridine ligand 89, the Kroehnke method was again used, despite difficulties found in purification of the methyl ligand 89. The same Kroehnke reagent 83 as in the 5’ substituted bipyridine synthesis was used, and reacted with crotonaldehyde 88. In the 5’ substituted 2,2’-bipyridine synthesis the methyl ligand 85 could easily be purified by a filtration silica column, however the 4’ substituted methyl ligand 89 stuck to the column and could not be eluted. Thus, in this case, the acid formation and subsequent methyl ester formation were performed crude, with the methyl ester ligand 91 purified prior to ruthenium(II) complexation. Ruthenium(II) complexation and subsequent deprotection afforded the 4’ monosubstituted acid Ru(II)(bpy)$_3$ complex 64.
7.3 Appendix III - Principle component analysis and linear discriminant analysis

A qualitative description of both principle component analysis (PCA) and linear discriminant analysis was presented in Chapter 4. This appendix presents some of the mathematics behind these techniques, using the Ru(II)(bpy)$_3$ complex-protein array as an example, where appropriate.

The aim of both of these analyses is to generate equations which give linear combinations of the data original data, e.g.

\[ X = a_1A + b_1B + \cdots + h_1H \]  
Eq. 7.23

Where A, B, ..., H are the luminescence responses from the different Ru(II)(bpy)$_3$ complexes and $a_1$, $b_1$, ..., $h_1$ are linear components used to generate the first principle component/linear discriminate, these are derived using the analysis. This equation can also be written in a vector format.

\[ X = w^T a \]  
Eq. 7.24

Where \( w = \begin{pmatrix} a_1 \\ b_1 \\ \vdots \\ h_1 \end{pmatrix} \), and \( w^T \) its transpose and \( a = \begin{pmatrix} A \\ B \\ \vdots \\ H \end{pmatrix} \).

These equations/ vectors project the data onto different axes, in the hope to have the maximum possible variance (in PCA) or the maximum discrimination of classes (in LDA).

7.3.1 Statistical definitions

Before using the more complicated mathematics it is necessary to define some statistical variables.

Mean  
\[ \mu_A = \frac{1}{n}(a_1 + a_2 + \cdots + a_n) \]  
Eq. 7.25

Variance  
\[ Var(A) = \frac{1}{n-1} ((a_1 - \mu_A)^2 + (a_2 - \mu_A)^2 + \cdots + (a_n - \mu_A)^2) \]  
Eq. 7.26

Covariance  
\[ Cov(A, B) = \frac{1}{n-1} ((a_1 - \mu_A)(b_1 - \mu_B) + \cdots + (a_n - \mu_A)(b_n - \mu_B)) \]  
Eq. 7.27

Note (by definition) \( Cov(A, B) = Cov(B, A) \)
7.3.2 Principal component analysis

Principle component analysis looks at addressing the variance in the data, without taking into account the classes.

Let the vectors $a, b, c ... x$ etc. be each of the individual protein replicates

$$
a = \begin{pmatrix} a_1 \\ a_2 \\ \vdots \\ a_8 \end{pmatrix}, \quad b = \begin{pmatrix} b_1 \\ b_2 \\ \vdots \\ b_8 \end{pmatrix}, \quad c = \begin{pmatrix} c_1 \\ c_2 \\ \vdots \\ c_8 \end{pmatrix} \text{ etc.}
$$

Where $a_1$ = the response of Ru(II)(bpy)$_3$ complex 1 in protein replicate a, $a_2$ = the response of Ru(II)(bpy)$_3$ complex 2 in protein replicate a, etc. for the 8 Ru(II)(bpy)$_3$ complexes and for all the protein replicates (b, c, etc.).

The mean of these vectors can be calculated to give a mean vector, $\mu$

$$
\mu = \begin{pmatrix} \mu_1 \\ \mu_2 \\ \vdots \\ \mu_8 \end{pmatrix}
$$

Where $\mu_1$ = the mean luminescence response of Ru(II)(bpy)$_3$ complex 1 in all the protein replicates, $\mu_2$ = the mean luminescence response of Ru(II)(bpy)$_3$ complex 1 in all the protein replicates, etc.

Using the mean vector it is possible to recenter the vectors $a, b, c, \text{etc.}$ around 0, by subtracting the mean vector, $\mu$

$$
a - \mu, b - \mu, c - \mu \text{ etc.}
$$

These new vectors can be combined to give a matrix, $X$, which contains all the data, centred about 0.

$$
X = \begin{pmatrix} a_1 - \mu_1 & b_1 - \mu_1 & \cdots & x_1 - \mu_1 \\ a_2 - \mu_2 & b_2 - \mu_2 & \cdots & x_2 - \mu_2 \\ \vdots & \vdots & \ddots & \vdots \\ a_8 - \mu_8 & b_8 - \mu_8 & \cdots & x_8 - \mu_8 \end{pmatrix}
$$

Using this data matrix, $X$, it is possible to define the covariance matrix, $C$

$$
C = \frac{1}{x-1}XX^T \quad \text{Eq. 7.28}
$$

Where $x$ is the number of protein replicates, and $X^T$ is the transpose of matrix $X$
This covariance matrix, \( C \), is symmetric (\( C = C^T \)) by definition.

As \( C \) is symmetric it has real eigenvalues (and corresponding eigenvectors) which fulfil the equation-

\[
Cw_i = \lambda_i w_i \quad \text{Eq. 7.29}
\]

Where \( \lambda_i \) = eigenvalue, and \( w_i \) = the corresponding eigenvector.

The solutions to this equation will give \( x \) eigenvectors and eigenvalues.

These eigenvalues can be put into magnitude order (\( \lambda_1 \geq \lambda_2 \geq \lambda_3 \ldots \geq \lambda_x \geq 0 \)). It may be that a small number (2 or 3) of the eigenvalues are much larger than the other eigenvalues, this means that these take the majority of the variation in the original data set. The corresponding (orthonormal) eigenvectors are the vectors \( w \) in Eq. 7.24 with the variables in the vector being the linear components \( (a_1, b_1, \ldots, h_1) \) in Eq. 7.23. These can be computed computationally.

### 7.3.3 Linear discriminant analysis

As seen above principle component analysis does not take into account the classes of the data, instead it keeps the spread in the data, this means that it may not be looking at components in the correct plane to discriminate different classes. This issue is addressed with linear discriminant analysis. Here the original data is project onto a multidimensional surface to increase the discrimination of the original data.

As with PCA, let the vectors \( a, b, c \ldots x \) etc. be each of the individual protein replicates

\[
a = \begin{pmatrix}
a_1 \\ a_2 \\ \vdots \\ a_B \\
\end{pmatrix}
\quad b = \begin{pmatrix}
b_1 \\ b_2 \\ \vdots \\ b_B \\
\end{pmatrix}
\quad c = \begin{pmatrix}
c_1 \\ c_2 \\ \vdots \\ c_B \\
\end{pmatrix}
\quad \text{etc.}
\]

However now, these samples \( a, b, c \) also have class labels \( y_a, y_b, y_c \) etc.

This means that we can define separate mean vectors for each class (i), \( \mu_i \), and we can take the total number of samples in class i, as \( M_i \). Such that-

\[
M = \sum_{i=1}^{C} M_i \quad \text{Eq. 7.30}
\]

Where \( C \) is the total number of classes.
From these parameters it is possible to define two scatter matrices, the within class scatter matrix ($S_w$) and the between class scatter matrix ($S_b$).

$$S_w = \sum_{i=1}^{C} \sum_{j=1}^{M_i} (y_j - \mu_i)(y_j - \mu_i)^T$$  \hspace{1cm} \text{Eq. 7.31}

$$S_b = \sum_{i=1}^{C} (\mu_i - \mu)(\mu_i - \mu)^T$$  \hspace{1cm} \text{Eq. 7.32}

Where $\mu$ is the mean of the whole data set- $\mu = \frac{1}{c} \sum_{i=1}^{c} \mu_i$ \hspace{1cm} \text{Eq. 7.33}

These scatter matrices can be transformed onto new planes ($W$) using the equations-

$$S_w = W^T S_w W$$  \hspace{1cm} \text{Eq. 7.34}

$$S_b = W^T S_b W$$  \hspace{1cm} \text{Eq. 7.35}

The aim of LDA is to find an optimal $W$ ($W^*$) such that $\det(S_b)/\det(S_w)$ is maximised, i.e. finding the minimum within class scatter to the maximum between class scatter.

$$W^* = \arg\max \left\{ \frac{W^T S_b W}{W^T S_w W} \right\}$$  \hspace{1cm} \text{Eq. 7.35}

This is found by finding a matrix $W^*$ whose columns are the eigenvectors ($w_i$) corresponding to the largest eigenvalues of the following equation-

$$(S_B - \lambda_i S_w)w_i = 0$$  \hspace{1cm} \text{Eq. 7.36}

These eigenvectors and eigenvalues can again be found computationally.
7.4 Appendix IV- DCC array studies with hydrazone functionalised Ru(II)(bpy)$_3$ complexes

The arrays discussed in Chapter 4, expand on previously reported luminescence arrays, using new types and combinations of molecules. However, recently the Waters group reported a study combining DCC and arrays, in order to distinguish different histone post-translational modification states. This brings together the ideas presented in Chapter 3 and Chapter 4. In Chapter 3 the development of hydrazone-based DCC was discussed; this is a combinatorial approach whereby a range of potential candidates is sampled in order to find ligands for a particular template (like a protein). The discussion of DCC presented in Chapter 3 was for the generation of high affinity protein ligands, by sampling a wide range of different potential candidates. However when using this approach, it could be that potent protein ligands are not generated, for example, if there is not a potent ligand within the range of potential candidates. Even if high affinity ligands are not generated, it could be that many low affinity ligands are present in the range of potential candidates. Therefore it could be that instead these low affinity ligands could be detected, and the ratios of the candidates present analysed as an array (Figure 7.1).

![Cartoon depiction of DCC array](image.png)

**Figure 7.1** Cartoon depiction of DCC array

The glycine hydrazide Ru(II)(bpy)$_3$ complex 49 was used (Scheme 7.3) in these studies; as discussed in Chapter 3 the hydrazones of this Ru(II)(bpy)$_3$ complex 50 were insoluble in DMSO/aqueous mixtures, and hence the hydrazone exchange could not be studied. However, for the array study, slightly lower concentrations were used and the starting point for these experiments made use of the water soluble hydrazide Ru(II)(bpy)$_3$ complex, rather than the insoluble hydrazone Ru(II)(bpy)$_3$ complexes 50.
Firstly, incubations were performed with 2 and 3 different aldehydes (4-carboxy benzaldehyde 44c, 2,4-dimethoxy benzaldehyde 44b and benzaldehyde 44a), with the hydrazide Ru(II)(bpy)₃ complex 49, aniline 45 and 3 different proteins (BSA, lysozyme and cyt c). After 16 hours incubation, attempts to separate the Ru(II)(bpy)₃ complexes formed from the proteins using protein concentrators (MWCO 5 kDa) were made, however due to the insolubility of the Ru(II)(bpy)₃ hydrazone complexes 46 they precipitated, staying with the protein, rather than filtering through to the flow through with the aldehydes and aniline. A similar problem was found to happen when protein incubations with the porphyrin DCLs were performed, as discussed in Chapter 3.

As the aim of these experiments was for the discrimination between proteins and not the generation of high affinity protein ligands, analysis of the flow through from the protein concentrator would yield information as to the aldehydes present, and therefore those not used to form hydrazones. As just the aldehydes were to be analysed, rather than the complex mixture of Ru(II)(bpy)₃ hydrazone complexes 50, it was possible to use a
wider range of aldehydes, and thus sample a much wider range of Ru(II)(bpy)$_3$ hydrazone complexes. To this end 10 different aldehydes (Figure 7.2) were used.

This experiment was performed with both 2 equivalents and 6 equivalents of each aldehyde 44 per hydrazide Ru(II)(bpy)$_3$ complex 49, and the aldehyde mixture in the protein concentrator flow through analysed by analytical HPLC. The peaks present at various different retention times were analysed, and the percentage of the total area used. This gave a fingerprint bar chart for the proteins (Figure 7.3) similar to those previously described for the luminescence arrays. Upon inspection of the bar charts very little discrimination between the proteins, was observed that can be discerned by eye with either the 2 or the 6 aldehyde equivalents data.

![Figure 7.3](image)

**Figure 7.3** Fingerprint bar charts for the DCC arrays with the glycine hydrazide Ru(II)(bpy)$_3$ complex 43 and 10 aldehydes, using 2 equivalents (a) and 6 equivalents (b) of aldehyde to hydrazide Ru(II)(bpy)$_3$ complex 43. Each bar represents the relative area under a peak at a particular retention time in the HPLC trace.

To see if protein discrimination could be achieved using this technique, LDA (Figure 7.4) was attempted, even though the fingerprints looked similar. This shows some clustering of the data points, and especially in the case of using 6 equivalents of aldehyde 44 shows that the data for the different proteins may be clustering separately, indicating that there is potential to use this technique for the discrimination of different proteins. However, more data would need to be obtained from many more replicates in order to determine if this is statistically significant, as well as controls without the Ru(II)(bpy)$_3$ hydrazide complex 49 present to see if it is the aldehydes reacting with the protein that cause these differences rather then the hydrazone-based DCC around the Ru(II)(bpy)$_3$ complex 49.
Figure 7.4 2-D LDA of the DCC array data with the glycine hydrazide Ru(II)(bpy)$_3$ complex 43 and 10 aldehydes 44, using 2 equivalents (a) and 6 equivalents (b) of aldehyde to hydrazide Ru(II)(bpy)$_3$ complex 43
7.5 Appendix V – Stabilisation of the p53 tetramerisation domain

Thus far the Ru(II)(bpy)$_3$ complexes presented have been studied as protein surface mimetics for use in PPI inhibition and protein sensing. However, another potential use of protein surface mimetics is PPI stabilisation. Previously guanidine functionalised calixarenes and peptides, and amine functionalised peptides have been used to stabilise the p53 tetramerisation domain.$^{212-215}$ It was thought that instead Ru(II)(bpy)$_3$ complexes may be capable of performing the same role.

7.5.1 The p53 tetramerisation domain

p53 is a tumour suppressor protein, which functions as a homotetramer.$^{216}$ It is an essential transcription factor for the cell cycle and apoptosis.$^{217}$ p53 mutations have been implicated in many human cancers, making it an interesting anti-cancer target.$^{217}$

Figure 7.5 X-ray crystal structure of the p53 tetramerisation domain, highlighting residue 337. a) Full structure, Arginine-337 shown in blue, b) Zoom in showing residue 337 in the wild-type protein (arginine, blue) and in the R337H mutant (red).

The tetramerisation of p53 is controlled by a short, 30-mer, region of the p53 protein, the tetramerisation domain.$^{218}$ Each monomer within the tetramerisation domain consists of an α-helix and a β-stand, which pack together to form the tetramer (Figure 7.5a). The surface of this tetramerisation domain of p53 is rich in acidic amino acid residues.

Alanine scanning mutation studies performed by the Fersht group showed which of the residues in the tetramerisation domain sequence were important for the tetramerisation,$^{218}$ implicating nine hydrophobic residues present in the core of the
tetramer. One of the amino acids implicated in this study was Arg-337; the R337A mutation resulted in a decreased melting temperature \((T_m)\) of 39.2 °C compared to the wild-type protein. A histidine mutation in this position is the most frequently inherited mutation affecting p53 tetramerisation\(^{219}\) and results in increased chances of tumour growth in organisms possessing this mutation.\(^{220}\) This R337H mutant of the p53 tetramerisation domain was chosen as a starting point to see if it was possible to stabilise the quaternary structure of p53, using Ru(II)(bpy)\(_3\) complexes (Figure 7.6).

![Figure 7.6 Cartoon depicting hypothesised binding of the Ru(II)(bpy)\(_3\) complexes to the p53 tetramerisation domain, resulting in stabilisation of the tetrameric structure](image)

7.5.2 Ru(II)(bpy)\(_3\) complexes and peptides

![Image of Ru(II)(bpy)\(_3\) complexes and p53 tetramerisation domain peptides](image)

**Figure 7.7** Ru(II)(bpy)\(_3\) complexes and p53 tetramerisation domain peptides. a) Ru(II)(bpy)\(_3\) complexes, b) Wild-type and R337H p53 peptides, showing the R337H mutation in red

A central 30-mer section of the p53 protein has been shown to be responsible for its tetramerisation,\(^{218}\) therefore, it was decided to use synthetic peptides (Figure 7.7b) rather than protein to form the p53 tetramers. Peptides have also been used in a previous study of calixarene stabilisers of the p53 tetramerisation domain.\(^{213}\) The use of peptides allowed
the purchase of two peptides, a wild-type (WT) and one possessing the R337H mutation, to allow a study of structural and binding differences between the two peptides with and without the Ru(II)(bpy)$_3$ complexes present. Using peptides allowed us to obtain the p53 tetramerisation domain quickly without the need to obtain a transcription vector or spend time optimising the protein preparation. Two amine functionalised Ru(II)(bpy)$_3$ complexes (35 and 81) (Figure 7.7a) previously synthesised were hypothesised to bind to the p53 tetramerisation domain, by analogy to lysine functionalised peptides which are known to bind, to the acidic surface of the p53 tetramerisation domain.221

7.5.3 Circular dichroism

Circular dichroism can be used to probe the secondary structure of peptides and proteins, showing their α-helical, β-sheet and random coil content. Therefore this technique was used to probe how structured the p53 peptides are. In order to establish if it is possible to use the Ru(II)(bpy)$_3$ complexes to stabilise the p53 tetramerisation domain peptide structures, thermal unfolding of the structured peptides was performed, and the ellipticity at 222 nm (corresponding to α-helical content) followed and converted to fraction unfolded (with 1 corresponding to the minimum magnitude ellipticity and 0 corresponding to the maximum magnitude ellipticity)

7.5.3.1 In phosphate buffer

![Figure 7.8 CD spectra in 5 mM sodium phosphate buffer, pH 7.5 a) CD spectra at 20 °C, b) Thermal melt, looking at ellipticity at 222 nm, corresponding to α-helical content. 100 μM peptide and 100 μM Ru(II)(bpy)$_3$ complex where appropriate](image)

Initially the CD spectra were recorded in 5 mM sodium phosphate, pH 7.5 buffer, to see if it may be possible to stabilise the p53 tetramerisation domain in a low concentration buffer. CD spectra of the two peptides alone at 20 °C showed significant α-helical content for both (Figure 7.8a), however there are differences in the spectrum, indicating
differences in secondary structure between the two peptides. Addition of 1 equivalent of Ru(II)(bpy)$_3$ complex 81 to the R337H peptide shows a difference in the CD spectrum, indicating some change in secondary structure. Addition of 4 equivalent of Ru(II)(bpy)$_3$ complex 81 to the R337H peptide, again showed differences in the spectrum, however much of this is likely to arise from the absorbance of the Ru(II)(bpy)$_3$ complex 81 which was found to distort the spectra at these concentrations.

Looking at the thermal melt profile (Figure 7.8b) it can clearly be seen that the structure of the R337H peptide is destabilised compared to the wild type peptide, reducing the melting temperature ($T_m$) from $\sim$52 °C to $\sim$29 °C. On addition of 1 eq. of Ru(II)(bpy)$_3$ complex 81 to the R337H peptide, there is a very small shift in the thermal melt profile, however the $T_m$ only shifts by $\sim$1 °C which is not particularly significant.

### 7.5.3.2 In water

The CD spectra in 5 mM sodium phosphate, pH 7.5 show that both the p53 peptides are structured, however the addition of Ru(II)(bpy)$_3$ complex 81 shows little change in the $T_m$ of the R337H peptide. Most previous work on synthetic stabilisers of the p53 tetramerisation domain had been performed in water,\textsuperscript{212,214,221,222} therefore attempts at the CD thermal melt study were also performed in water.

The CD spectra and thermal melt profiles of the wild type and R337H p53 peptides in water look similar to that in 5 mM phosphate buffer (Figure 7.9), with the wild-type peptide structure being stabilised by $\sim$10 °C in water compared to phosphate buffer. Addition of 1 eq. of Ru(II)(bpy)$_3$ complex 81 (Figure 7.9a and c) looks to have increased the $T_m$ of the R337H peptide by $\sim$10 °C indicating stabilisation of the structure while the $T_m$ of the wild-type peptide has been decrease by a similar amount, indicating destabilisation the structure. The potentially different responses of the Ru(II)(bpy)$_3$ complex to the two peptides indicate that it may be binding to the p53 tetramer in the region of the mutation, disrupting the interactions causing tetramerisation in the case of the wild-type peptide, and enhancing those interactions in the case of the R337H mutant peptide. Spectra with Ru(II)(bpy)$_3$ complex 35 indicate similar results (Figure 7.9b and d), however the data is a lot noisier, and should be repeated.
Figure 7.9 CD spectra in water a) CD spectra with Ru(II)(bpy)$_3$ complex 81 at 20 °C, b) CD spectra with Ru(II)(bpy)$_3$ complex 35 at 20 °C, c) Thermal melt profile with Ru(II)(bpy)$_3$ complex 81, d) Thermal melt profile with Ru(II)(bpy)$_3$ complex 35. All spectra taken in water, neutralised by addition of 1 mM HCl or 1 mM NH$_4$OH, with 100 μM peptide and 100 μM Ru(II)(bpy)$_3$ complex where appropriate.

7.5.4 Conclusions and future work

Preliminary data, using CD thermal melt profiles, has been obtained, indicating that the Ru(II)(bpy)$_3$ complexes 35 and 81 can stabilise structure of the R337H mutant peptide of the p53 tetramerisation domain. These studies also indicate that these Ru(II)(bpy)$_3$ complexes also destabilise the structure of the wild-type peptide of the p53 tetramerisation domain. This indicates that the Ru(II)(bpy)$_3$ complexes can be used as PPI stabilises, as well as PPI inhibitors.

In order to establish if the effects seen are valid, it is necessary to repeat the CD data, particularly for Ru(II)(bpy)$_3$ complex 35 in order to generate better thermal melt profiles. If the data does prove reproducible, further experiments will need to be performed in order to show: i) that it is indeed tetramers that do form, ii) an orthogonal experiment to show the stabilisation, iii) the stoichiometry of binding, iv) the binding affinity and v) the location of the binding site. Potential platforms for determining these factors include
native mass spectrometry, size exclusion chromatography, proteolytic digestion, luminescence anisotropy and protein NMR. Further work could also be performed to further destabilise the p53 tetramerisation domain, so that it is not structured at 20 °C, this would enable us to see if it is possible to induce protein structure on an unstructured peptide by addition of synthetic molecules.
8 References


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