SYNTHESIS AND CHARACTERISATION OF
PLATINUM(II) AND RUTHENIUM(II) POLYAMIDE
CONJUGATES

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STATEMENT OF AUTHENTICITY

This Thesis is submitted in partial fulfilment of the requirements for the postgraduate award Doctorate of philosophy at the University of Western Sydney; School of Biomedical and Health Sciences. The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not previously submitted this material, either in whole or in part at any other institution.

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ABSTRACT

This thesis reports the synthesis and characterisation of two novel metallo-polyamide conjugates; LLSP4-Pt and LLSP4-Ru. Several synthetic strategies were employed in the development of the polyamides, beginning with solution phase chemistry. Construction of the polyamide was attempted via chain elongation with sequential additions of pyrrole monomers, however, solution phase methods were unable to achieve the coupling of four consecutive pyrrole rings. Solid phase chemistry, using a peptide synthesiser, was then used as an alternative. Synthesis using solid phase chemistry required the production of the monomeric building block Fmoc-py-COOH using adaptations of various literature methods and was successfully characterised by $^1$H NMR. The proof-of-concept molecule LLSP4-DPA was the first polyamide made using Fmoc-$\beta$-Alanine-OH-WANG resin although several impurities from the cleaving agent 3-dimethylaminopropylamine persisted despite attempts at purification. The solid phase support was then changed to chlorotrityl resin and was used to synthesise the precursor polyamide LLSP4 with high purity. LLSP4-Pt was then successfully made using chlorotrityl resin and characterised by NMR ($^1$H and $^{195}$Pt), ESI-MS and elemental analysis. The ligands dpq, 4-CO$_2$H-phen and intermediates thereof, were successfully synthesised and used in the production of the ruthenium conjugate. The precursor LLSP4-(4-CO$_2$H-phen) was made using the same solid phase techniques employed for LLSP4-Pt. Coordination of [Ru(dpq)$_2$Cl$_2$] to LLSP4-(4-CO$_2$H-phen) was afforded by heating in EtOH producing LLSP4-Ru which was characterised by $^1$H NMR and ESI-MS. Preliminary studies showed that upon addition of DNA to LLSP4-Ru a large increase in fluorescence is seen which suggests an intercalative binding mode. DNA binding studies for LLSP4-Pt were conducted using CD based titrations with ct-DNA. The binding constant of LLSP4-Pt was found to be 4.1 $\times$ 10$^5$ M$^{-1}$ with a binding site size of 4 base pairs. The ability LLSP4-Pt to form coordinate covalent bonds with guanosine nucleosides was investigated and monitored by $^1$H NMR. Incubation of guanosine with LLSP4-Pt shows a new resonance for H1' is observed at 5.98 ppm while two new resonances for H8 are observed at 8.78 and 8.79 ppm.
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ABBREVIATIONS

4-CO$_2$H-4'-Mebpy: 4-Carboxy-4-methyl-2,2'-bipyridine
4-CO$_2$H-phen: 4-Carboxy-1,10-phenanthroline
4-Me-phen: 4-Methyl-1,10-phenanthroline
9-AA: 9-Aminoacridine
A: Adenosine
ACN: Acetonitrile
AH: Amphipathic helix
ASCII: American standard code for information interchange
AVA: Aminovaleric acid
C: Cytosine
CAM-DR: Cell-adhesion mediated-drug resistance
CD: Circular dichroism
cis-DDP: cis-Diamminedichloroplatinum(II) or cisplatin
CPI: Cyclopropapyrroloindole
ct-DNA: Calf thymus DNA
d$_6$-DMSO: Deuterated dimethyl sulphoxide
d$_7$-DMF: Deuterated dimethyl formamide
DCM: Dichloromethane
DIEA: N,N-diisopropylethylamine
di-Pt: [{Pt(NH$_3$)$_2$Cl$_2$}$\mu$-dppz]$^{2+}$
DMF: N,N$'$/dimethylformamide
DNA: Deoxyribonucleic acid
DNA$_f$: Free DNA binding site
DNA-PK: DNA-dependant protein kinase
dpppz: Dipyrido[3,2-a:2'3'-c]phenazine
dpq: Dipyrido[3,2-a:2'3'-c]quinoxaline
dpqC: Dipyrido[3,2-a:2'3'-c](6,7,8,9-tetrahydro)phenazine
Dst: Distamycin

*E. coli*: *Escherichia coli*

EBS: Ets binding site

EDTA: Ethylenediaminetetraacetic acid

en: 1,2-Diaminoethane

ESI-MS: Positive ion electrospray ionisation mass spectra

FDA: Food and Drug Administration

FID: Fluorescent intercalator displacement

FIDs: Free induction decays

Fmoc-Cl: 9-Fluorenylmethylchloroformate

Fmoc-py-COOH: 4-(Fmoc-amino)-1-methylpyrrole-2-carboxylic acid

G: Guanine

Gly: Glycine

GSH: Glutathione

HBTU: O-Benzotriazole-\(N,N,N',N'\)-tetramethyluroniumhexafluorophosphate

HIV-1: Human immunodeficiency virus-1

HOBt: \(N\)-hydroxybenzotriazole

Hox: Homeobox

Hp: 3-Hydroxypyrrole

HPLC: High performance liquid chromatography

Im: Imidazole

\(K_b\): Binding constant

KBr: Potassium bromide

LD: Linear dichroism

LLSP4: Linear linker solid phase 4-ring

\(M_b\): Bound metal complex

\(M_f\): Free metal complex

MT: Metallothionein
NER: Nucleotide-excision-repair pathway
NMP: N-methylpyrrolidinone
NMR: Nuclear magnetic resonance
NOESY: Nuclear overhauser effect spectroscopy
Nt: Netropsin
Phen: 1,10-Phenanthroline
Phendo: 1,10-Phenanthroline-5,6-dione
polII holoenzyme: RNA polymerase II holoenzyme
Py1: 2-Trichloroacetyl-1-methylpyrrole
Py2: 4-Nitro-2-trichloroacetyl-1-methylpyrrole
Py3: tert-Butyl 4-nitro-1-methylpyrrole-2-carboxylate
Py4: tert-Butyl 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate
Py5: 4-[(9-Fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid
PyBOP: Benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate
PyPyPyCO₂H: 1-Methyl-4-(1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl)-amino)-1H-pyrrole-2-carboxylic acid
R: Metal complex:DNA ratio
STF1: Synthetic transcription factor 1
T: Thymine
TALE: Three amino acid loop extension
TBTU: O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
TEA: Triethylamine
TFE: Trifluoroethanol
THF: Tetrahydrofuran
TLC: Thin layer chromatography
$T_m$: Melting temperature
$trans$-DDP: $trans$-Diamminedichloroplatinum(II) or transplatin
UV-Vis: Ultraviolet-visible
$\beta$-ala: $\beta$-Alanine
$\gamma$-ABA: $\gamma$-Aminobutyric acid
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1.1 Deoxyribonucleic Acid

The elucidation of the structure of deoxyribonucleic acid (DNA) by James Watson and Francis Crick in 1953 was recognised by many as one of the most important scientific discoveries of the 20th century. The article published in *Nature* was the first to describe correctly the unique structure of DNA.¹ This discovery was only achieved through the work of Rosalind Franklin, a gifted crystallographer whose efforts facilitated Watson and Crick’s discovery, during her time at King’s College, London. Franklin’s research into DNA X-ray crystallography led to the well recognised photograph 51 (Figure 1.1).² The black striped pattern extending from the centre of the X-ray provided evidence to the double helical structure of DNA.

![Figure 1.1](image)

**Figure 1.1** X-ray diffraction pattern of DNA exposed on photograph 51.

Maurice Wilkins, a collaborator with Franklin on the DNA project, showed photograph 51 to Watson in a brief meeting. Watson and Crick recognised the structural implications and this allowed them to lay claim to the discovery only months before fellow researcher in the field Linus Pauling. Pauling had earlier proposed a structure for DNA consisting of three intertwined chains with the DNA bases extruding from the helical structure.³ ⁴ This proposition was rejected due to several flaws in the data. The X-ray structure provided by Pauling was of the sodium salt, not the free acid form, which did not provide the hydrogen bonds that participate in holding the structure together, and secondly, it was noted that some van der Waals distances in the structure appeared to be too small.
Using the information acquired from photograph 51 Watson and Crick put forward a model consisting of two helical chains coiled about the same axis, with their bases residing within the helix and the phosphate groups on the outside. Stability of the helix in this model was afforded by hydrogen bonding between the base pairs which had special pairing rules; one of the bases must be a pyrimidine {a heterocyclic compound with a benzene ring containing nitrogen atoms at positions 1 and 3; thymine (T), cytosine (C)} and one a purine {a heterocycle with a pyrimidine fused to a imidazole (Im) group; adenosine (A), guanine (G)} (Figure 1.2). To their credit Watson and Crick were also able to deduce that this pairing system could afford a copying mechanism for DNA, which at the time was being debated due to the lack of diversity provided by merely four bases.

Figure 1.2 Hydrogen bonding between the complementary base pairs A-T and G-C, the R groups represent the link to sugar phosphate backbone.

1.1.1 DNA Structure

DNA is a polynucleotide composed of repeating units of a phosphate group, a ribose sugar and a nucleobase. Although composed of bases, the presence of the phosphate groups defines it as an acid, hence the term nucleic acid. The sugar group found in DNA is 2-deoxy-\textit{D}-ribose while a similar form of the sugar, \textit{D}-ribose, is the constituent of ribonucleic acid or RNA. The polynucleotide structure of DNA is produced through the formation of
phosphodiester bonds of the C'$_3$ hydroxyl of one nucleotide unit to the C'$_5$ phosphate of an adjacent nucleotide. Through variation of the base present in these nucleotides a specific sequence is achieved in the DNA chain. This sequence is used as the storage of hereditary information (genes) responsible for encoding the machinery critical for cellular function.

In addition to Watson and Crick’s model of right-handed DNA (B-DNA) it is known that the anti-parallel strands of nucleic acid can assume several other conformations. These forms of DNA include A-, B- and Z-DNA conformation (Figure 1.3). B-DNA is regarded as the native form and is found in both eukaryotic and bacterial organisms.

![Three forms of DNA](image)

**Figure 1.3** Three forms of DNA. The structures shown are a A-DNA, b B-DNA and c Z-DNA, displaying the varying physical properties. (Structure generated by Hyper Chem™)

The stability of B-DNA is achieved by complementary base pairing between the two intertwined polynucleotide chains. The complementary bases are A-T and C-G, known as Watson and Crick base pairs. These hydrogen bonds are not the only forces stabilising the double helix. The base pairs of each helix are able to stack upon each other, a phenomenon
known as base pair stacking whereby the $\pi$-orbitals of the aromatic rings overlap and further stabilise the DNA strand. Due to the hydrophobic nature of DNA bases they are stacked inside of the helix perpendicular to the helical axis under physiological conditions. The ribose phosphate backbone is hydrophilic and therefore favours exposure to the aqueous environment. Under normal physiological conditions, B-DNA has a helical rise of 3.4 Å per base pair and consecutive bases rotate an average of 36° around the helical axis. This rotation causes the structure to repeat itself every ten base pairs. The glycosidic link between deoxyribose and the DNA base is asymmetrical leading to the formation of two grooves. These grooves of different widths but almost equal depths, are known as major and minor grooves. The major and minor grooves display widths of 11.7 and 5.7 Å respectively.

1.2 Metal Complexes as DNA Probes

The use of metal complexes as probes of DNA topology has been an active area of research for the past several decades. Interactions between DNA and metal complexes occur via covalent/coordinate bond formation and/or reversible intermolecular associations.

Reversible DNA binding complexes do not form covalent bonds, instead they exist bound to DNA as part of an intricate equilibrium. The forms of reversible intermolecular associations consist of: external binding, groove binding and intercalation. It must be noted some complexes are capable of more than one mode of binding, eg. proflavine.

1.2.1 Binding Modes

1.2.1.1 Coordinate Covalent Binding

In general, coordinatively unsaturated metal complexes bind to DNA through coordinate covalent bonds. Square planar complexes such as $[\text{PtCl}_4]^{2-}$ and cis-$[\text{Pt(NH}_3)_2\text{Cl}_2]^{2+}$ bind to DNA via coordination to the N7 of guanine and can form interstrand and intrastrand cross-linking. Complexes such as $[\text{Co(H}_2\text{O})_6]^{2+}$ are also capable of binding to the N7 of guanine, while other octahedral complexes including $[\text{Ru(phen})_2\text{Cl}_2]$ (phen = 1,10-phenanthroline)
bind covalently and show some enantiomeric preference.\textsuperscript{6, 7} There are complexes also able to bind via intercalation in addition to covalent bond formation such as $\textit{cis}$-$\text{[Pt(NH}_3)_2(N9-9-AA)Cl]}(\text{NO}_3)$ (where 9-AA = 9-aminoacridine) and $\text{[Cu(phen)_2]}^{2+}$\textsuperscript{8, 9}

1.2.1.2 External Binding

External binding is primarily due to electrostatic interactions between the negatively charged phosphate backbone and positively charged ions. Cations such as $\text{[Mg(H}_2\text{O)}_6]}^{2+}$ and $\text{Na}^+$ aggregate on the exterior surface of DNA helix, while planar compounds like proflavine can bind externally by stacking along the double helix.\textsuperscript{10-12} More complex ions such as metal complexes with octahedral or square planar geometries can bind electrostatically within the major or minor grooves of DNA. For instance, the octahedral metal complex $\text{[Co(en)}_3]^{3+}$ ($\text{en} = 1,2$-diaminoethane) binds to DNA in the major groove, whereas the square planar complex $\text{[Pt(en)}_2]^{2+}$ binds in the minor groove.\textsuperscript{13}

1.2.1.3 Groove Binding

As the name suggests it is the reversible interaction of molecules that occurs with either the major or minor groove of DNA. This binding is composed mainly of electrostatic interactions, hydrogen bonding and van der Waals forces. The topology of the major and minor grooves differs significantly in size hydration, electrostatic potential and position of hydrogen bonding sites. Small organic molecules, usually crescent shaped with a long thin profile, such as Hoechst 33258 (Figure 1.4) prefer the minor groove. Hoechst is a minor groove binder commonly used in fluorescence microscopy that emits a bright blue colour when bound to DNA.\textsuperscript{14} This binding is stabilised by the close van der Waals interactions of the narrower regions of the minor groove found at AT rich sequences. The major groove binds larger compounds in preference, such as those containing large organic chains and proteins.
1.2.1.4 Intercalation

Intercalation is the insertion of a planar aromatic moiety between the base pairs of DNA. First described by Lerman, this mode of binding is stabilised by the overlap of π-orbitals from the ligand and the nucleobase in addition to any electrostatic forces present.\textsuperscript{15} Intercalation increases the distance between the adjacent bases causing distortion of the duplex and conformational changes in DNA structure. This distortion is compensated for via changes in the phosphate backbone, most commonly unwinding and lengthening of the duplex is observed. The sugar-phosphate torsional angles change to accommodate the intercalator. Intercalation is a common mode of association for small planar molecules such as acridine and proflavine (Figure 1.5) that is facilitated from either the major or minor groove.

![Figure 1.4](image1.png)

**Figure 1.4** The DNA groove binder Hoechst 33258.

![Figure 1.5](image2.png)

**Figure 1.5** DNA intercalators: \(\textbf{a}\) acridine, \(\textbf{b}\) proflavine.

Acridine, a natural coal extract, is used for the production of dyes and as a precursor of several drugs. A derivative of acridine is proflavine, a mutagen that has been utilised in molecular biology.\textsuperscript{16} Proflavine has been shown to bind to DNA via two modes.\textsuperscript{12, 16, 17} At
low concentrations there is classical intercalation between successive DNA base pairs while at high concentrations a weak external binding to the phosphate groups is seen.

1.2.2 Ruthenium(II) Polypyridyl Complexes

The initial venture into ruthenium polypyridyl complexes began with the celebrated Australian chemist Professor Francis Patrick Dwyer in the early 1950s. Dwyer was captivated with the potential of inert metal complexes within biological systems. His initiative was not widely shared and his foray into this area of research late in his career was considered by many a futile endeavor. Fortunately his work did prove to be very valuable and provided a foundation to the understanding of metal complexes in biological systems.

Dwyer believed that polypyridyl ligands such as phen might share some of the characteristics of alkaloids (biologically active heterocyclic agents that contain nitrogen within their ring systems). Adamant this was the case, Dwyer persuaded his colleagues at the time to taste the complexes (after doing so himself) and noted the characteristically bitter taste shared by alkaloids. Dwyer expanded his research and began investigating the effects of the $\Delta$ and $\Lambda$ isomers of $\text{tris(phenanthroline)ruthenium(II)}$, $[\text{Ru(phen)}_3]^{2+}$, on animals. In these studies it was found the $\Delta$ enantiomer was twice as toxic as the $\Lambda$ isomer, and additionally the complex had passed through the animals intact. Prior to these experiments it was undetermined whether activity was from the complex itself or from disassociated ligands, despite knowledge of their inertness under boiling acidic or alkali conditions. This observation therefore reinforced the idea that the intact complex was primarily responsible for activity. The work pioneered by Dwyer and his peers showed great potential with the promise of practical purposes, such as drug development in the future.

Prior to research on ruthenium as DNA binding molecules, the interest in the field focussed on other transition metals. In the early 1980s resurgence in ruthenium polypyridyl complexes began to emerge due to their unique characteristics. The chemical stability, excited state reactivity and lifetime, redox properties and luminescence emission of this class of
complexes had attracted a large group of the researchers stimulating several branches of pure and applied chemistry. These complexes were extensively investigated as conversion and storage systems of solar energy. It was discovered Ru(II) polypyridyl compounds with carboxylic acid groups have the best solar to electric power conversion efficiency.\(^\text{20}\) Their use as photogalvanic cells could not be achieved as high energy light was required for the wide band-gap of the photostable semiconductors, making such efforts impractical.\(^\text{*}\) The design and synthesis of metal complexes with polypyridyl ligands for use as DNA probes or chemical photonucleases also grew into a highly active area of research. Initial efforts in this area concentrated on the DNA binding of platinum,\(^\text{21-23}\) copper,\(^\text{24, 25}\) and zinc\(^\text{26}\) complexes. The inherent nature of transition metal complexes allows for variation of both metal and ligand while maintaining similar characteristics. Eventually, the focus on polypyridyl complexes of ruthenium(II) grew because of their DNA binding potential and luminescent properties.

1.2.2.1  \textit{Tris(phenanthroline)ruthenium(II)}

A simple and thoroughly investigated polypyridyl metal complex in terms of DNA binding is the chiral compound [Ru(phen)\(_3\)]\(^{2+}\) (Figure 1.6). Initially, the binding of this complex was proposed by Barton \textit{et al.} to be primarily via intercalation,\(^\text{27}\) with groove binding later added as a secondary mode.\(^\text{28}\) Definitive evidence for both modes of binding was lacking as each assumption was based upon inconclusive spectroscopic measurements and the debate about where binding was taking place soon ensued.

Barton and co-workers later proposed that both enantiomers of [Ru(phen)\(_3\)]\(^{2+}\) display two DNA binding modes: the $\Lambda$-isomer favoring binding on the DNA surface and the $\Delta$-enantiomer preferring intercalation.\(^\text{27-30}\) This theory was countered by Hiört and co-workers who questioned certain aspects of the binding mechanism.\(^\text{31}\) Hiört argued that the evidence supporting intercalation was questionable and other factors could be responsible for these

\(^*\) Photogalvanic cells convert and store solar energy into electrical energy while semiconductors control the electrical conductivity. The ability for electrons in a semiconductor to be excited from the valence band to the conduction band to produce an electrical current depends on the size of the band gap between the bands.
observations. The unwinding of supercoiled circular DNA upon binding of the complexes, did not confirm intercalation was responsible, as many non-intercalative groove binding compounds are also capable of helical relaxation. Based on linear dichroism experiments Hiört disputed that the angular orientation of the complex did not correspond with classical intercalative geometry. An angle of 70º was obtained for the Δ-enantiomer in relation to the DNA axis, while the corresponding Λ-enantiomer bound at 50º. This suggested the Δ-enantiomer did not intercalate as an angle of 55º was required, in contrast, the Λ-enantiomer possessed an orientation more consistent with intercalation. These findings raised doubts about the theory proposed by Barton; the new data suggested that neither of the enantiomers bound via intercalation and that their binding differed in geometry. For the Λ–isomer it was suggested one chelate wing points into the middle of the major groove while stability is achieved with the ancillary ligands interacting with the phosphate groups of DNA. Conversely, the Δ-complex, was suggested to bind with two chelate wings pointing into the groove and interacting with the base pairs, known as facial orientation.

![Figure 1.6](image)

**Figure 1.6** Enantiomers of tris(phenanthroline)ruthenium(II): a Λ-[Ru(phen)₃]²⁺, b Δ-[Ru(phen)₃]²⁺.

Viscosity measurements by Chaires and coworkers provided evidence that neither isomer binds via classical intercalation. The criteria for intercalation, as described by Lerman, requires the lengthening of the DNA helix upon insertion of the aromatic moiety between the
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base stack.\textsuperscript{15} Chaires was able to show that neither isomer lengthened short, rodlike DNA, a classical indicator of intercalation. In addition, the known groove binder Hoechst 33258 caused no changes in DNA viscosity, demonstrating groove binding does not necessarily lengthen DNA. It was concluded that the $\Lambda$-enantiomer behaves much like Hoechst 33258, binding in one of the helical grooves without distortion of DNA structure. Conversely, the $\Delta$-enantiomer decreases DNA viscosity. Previous reports of such effects by stereoselective DNA binding ligands were recorded by Kapicak and Gabbay.\textsuperscript{33} This anomaly was explained by a model in which the compounds were partially intercalated, with the aromatic moiety not capable of full insertion between the base pairs. As a result of this binding, a kink in the helix is formed that effectively reduces its length and consequently the viscosity.

It had been assumed by many in the field that ruthenium complexes would have to be major groove binders as it was thought that the minor groove could not accommodate such bulky molecules. A nuclear magnetic resonance (NMR) study on the binding of $[\text{Ru(phen)}_3]^{2+}$ to a short DNA duplex by Eriksson \textit{et al.} provided unambiguous evidence for minor groove binding.\textsuperscript{34} Experiments showed no nuclear overhauser effect spectroscopy (NOESY) cross peaks between aromatic/imino protons of the major groove and the phen protons. Eriksson was able to strengthen evidence against an intercalative mode of binding. Titration of the oligonucleotide with $[\text{Ru(phen)}_3]^{2+}$ found no reduction in DNA sequential connectivity cross peaks, indicating that no separation of the base pairs was occurring. Also, the binding kinetics were rapid and no line broadening observed, unlike intercalators where exchange kinetics are slow resulting in broadening of DNA protons.

Studies by Rodger \textit{et al.} have provided evidence for the dependence of metal complex:DNA ratio on binding mode.\textsuperscript{35} The effect of saturation, whereby all DNA sites are occupied, was investigated by circular dichroism (CD), linear dichroism (LD) and normal absorption spectroscopy, in addition to computer modeling. It was proposed $\Lambda-[\text{Ru(phen)}_3]^{2+}$ favors partial intercalation in the major groove at all mixing ratios. For $\Delta-[\text{Ru(phen)}_3]^{2+}$, at low metal complex:DNA ratios ($R$), minor groove binding is preferred whilst adopting a facial
orientation. At high $R$, a “slotted” mode whereby a single chelate is orientated along the minor groove becomes more favorable with some partial intercalation in the major groove also occurring.

It is apparent there has been little consensus about the binding mode of $[\text{Ru}(\text{phen})_3]^{2+}$ with several theories to explain its exact nature. More evident is the probability of multiple binding modes for this complex under varying conditions making the assignment increasingly more complicated. Nonetheless, the next key step in the development of Ru-polypyridyl complexes was to improve DNA affinity and create a more distinctive mode of binding.

**1.2.2.2 Ruthenium Intercalators**

Despite the favorable characteristics of Ru-polypyridyl complexes such as $[\text{Ru}(\text{phen})_3]^{2+}$, these complexes were incapable of classical intercalation and possessed weak binding affinity at best ($\Delta$: $K = 4.9 \pm 0.3 \times 10^4 \text{ M}^{-1}$, $\Lambda$: $K = 2.8 \pm 0.2 \times 10^4 \text{ M}^{-1}$).\(^{32}\) The planar aromatic moiety of the phen ligands did not complement the structure of DNA for full insertion within the base stack. Revision of the design, by extending the aromaticity of a single ligand, heralded the development of ruthenium trispolypyridyl complexes that were capable of complete intercalation. The ligand dipyrido[3,2-\text{a}:2'3'-\text{c}]phenazine (dppz) (Figure 1.7) first synthesised by Dickerson,\(^{36}\) was used to research the photophysical properties of ruthenium complexes for the development of galvanic cells.

Although initially synthesised for electrochemical, optical, and photophysical measurements, $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ possessed a structure well suited as a metallointercalator.\(^{37,38}\) In 1990, the complex was adopted by Barton and coworkers as a DNA probe with novel luminescence characteristics.\(^{39}\) Their study reported $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ as a molecular “light switch” for DNA, showing no photoluminescence in aqueous solution but intense fluorescence in the presence of DNA. The complex also showed high affinity for DNA ($K_b \geq 10^6 \text{ M}^{-1}$) with comparable binding for both A-T and G-C-rich sequences. These measurements showed that
in its ground and excited states charge transfer occurs from the metal centre to the phenazine ring where protonation of the nitrogen atoms occurs. In addition, it is proposed that when excited, this complex is able to transfer electrons through DNA to an acceptor molecule. The data obtained from these experiments offer a better understanding of redox mechanisms in biological processes such as electron transfers over large distances within metalloproteins.

The development of ruthenium polypyridyl intercalators has for the most part looked at modulation of the aromatic ring system. In addition to phen and dppz other intercalating ligands include dipyrido[3,2-a:2'3'-c]-quinoxaline (dpq)\textsuperscript{40, 41} and dipyrido[3,2-a:2'3'-c](6,7,8,9-tetrahydro)phenazine (dpqC).\textsuperscript{37-39, 41} It is well known that the number of aromatic rings fused concurrently increases the affinity of a intercalator for DNA. Therefore, in terms of binding strength dppz > dpqC > dpq > phen (Figure 1.7).

![Figure 1.7](image1.png)

**Figure 1.7** Structures of common intercalating ligands: a phen b dpq, c dpqC and d dppz.

The ligand dpq has recently been used by the Group of Aldrich-Wright to develop novel dinuclear complexes such as \([\{\text{Ru(dpq)}_2\}_2(\mu\text{-phen-3-SOS-3-phen})\]\textsuperscript{4+} (Figure 1.8).\textsuperscript{42} These dimers, linked via a flexible dithiol linker, consist of two bis-dpq ruthenium complexes capable of intercalation. The complexes studied had varying positions of linker attachment to the phen ligand (3, 4, 5) which produced slightly different distances between the metal centres. These complexes were observed to have a high affinity for DNA with binding

\[1\] Protonation of the phenazine nitrogen in aqueous solution efficiently quenches luminescence.
constants \((K_b)\) in the range of \(10^7 \text{M}^{-1}\). These binding affinities for the symmetrical dimers are a factor of 1000 times greater than that of the monomeric equivalent \([\text{Ru(dpq)}_2(\text{phen})]\text{Cl}_2\). The binding site sizes of these complexes were determined and found to span 4-8 base pairs, which is larger than the number of sites these mononuclear complexes span. Melting experiments of these dimers on a short duplex of DNA were used to elucidate the affect on thermal stability. The resulting \(\Delta T_m\) values varied between 9-20 °C, an indicator of intercalation and a strong affinity for DNA.

![Structure of the dinuclear complex \(\{\text{Ru(dpq)}_2\}_2(\mu\text{-phen-3-SOS-3-phen})\]^{4+}.](image)

**Figure 1.8** Structure of the dinuclear complex \(\{\text{Ru(dpq)}_2\}_2(\mu\text{-phen-3-SOS-3-phen})\]^{4+}.

### 1.2.3 Platinum Metal Complexes

Design and synthesis of platinum metal complexes as DNA binders is also an active area of research. The mechanisms by which these metal complexes exert their biological effectiveness are not fully understood, however, their various interactions with DNA are well documented. Although highly active, the indiscriminate binding of platinum based therapeutics leads to severe negative side effects as seen with *cis*-diamminedichloroplatinum(II) or cisplatin (*cis*-DDP).

#### 1.2.3.1 *cis*-Diamminedichloroplatinum(II)

The interest in platinum(II) complexes began in 1964, in an electrochemical study involving the effects of electric fields on *Escherichia coli* (*E. coli*) growth rates.\(^{43}\) It was discovered
that \textit{E. coli} cell division was arrested in the presence of the platinum electrodes although the cells continued to grow. Due to an interaction with the medium, \textit{cis}-DDP (Figure 1.9) was being deposited into the solution and found to be the causative agent. Further investigation showed that generation of \textit{cis}-DDP, in addition to \textit{trans}-diamminedichloroplatinum(II) (\textit{trans}-DDP) (Figure 1.9), was due to the electrolysis of the platinum electrodes.\textsuperscript{44} After extensive tests and clinical trials, \textit{cis}-DDP was approved by the FDA in 1979 and has been recognised as one of the most potent anticancer drugs available despite the negative side effects such as nephrotoxicity and neurotoxicity.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{cis-trans-ddp.png}
\caption{Structure of the anticancer drug \textbf{a} \textit{cis}-DDP and \textbf{b} \textit{trans}-DDP.}
\end{figure}

The mechanism of activity of \textit{cis}-DDP has been exhaustively studied although debates of the exact nature of action are on going.\textsuperscript{45} \textit{cis}-DDP contains two labile chlorine groups, which, in an aqueous environment where chloride ion concentration is low, undergo sequential hydrolysis.\textsuperscript{46} Following hydrolysis, \textit{cis}-DDP forms a positively charged species, which is drawn electrostatically to the negatively charged DNA backbone. Here, coordination adducts form preferentially with the N(7) of guanine, which has the lowest redox potential of the four nucleobases. The formation of these adducts can occur either via inter- or intrastrand crosslinks, of which, the intrastrand crosslink is the predominant lesion formed. The most abundant of these adducts formed are the 1,2-d(GpG) intrastrand crosslinks\textsuperscript{†}, encompassing approximately 65\% of adduction.\textsuperscript{47} The remaining products consist of the 1,2-d(ApG) and 1,3-d(GpG) intrastrand crosslinks (Figure 1.10) and the 1,2-d(GpG) interstrand crosslink.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{crosslinks.png}
\caption{Adduct nomenclature: The first two numbers are the positions of the adduct formations while the nucleotide abbreviations show which bases are involved. For instance, 1,2-d(GpG) (intrastrand) indicates that platinum is coordinating to a guanine residue on position 1 and to a guanine on position 2 (the adjacent base pair) on the same strand of DNA.}
\end{figure}
While there is evidence that interstrand adducts are capable of causing mutations in the genome, it is well known that the intrastrand 1,2-d(GpG) adducts are largely responsible for *cis*-DDP activity. Initial conventions on *cis*-DDP activity favoured the interstrand crosslinks as the primary mechanism of activity. This was readily believed as it seemed logical that an adduct that binds two strands of DNA together could easily interfere in normal cellular processes such as replication or transcription. When direct measurements on adduct populations revealed intrastrand crosslinks account for 80-90% of total adducts, this view shifted in favour of the intrastrand crosslink as the critical lesion. Upon intrastrand platination of 5'-GpG-3' a dihedral angle of 32° is formed between the two guanines. This “kink” in DNA causes structural distortions to the extent where the DNA helix unwinds.

**Figure 1.10** Crystal structure demonstrating the 1,3-d(GpG) intrastrand crosslink.48
13°. These distortions of DNA structure are not limited to intrastrand lesions. The binding of *cis*-DDP to 5'-GpG-3' via interstrand crosslinks accounts for 5-10% of total lesions formed. The effect of these structural distortions on polymerase migration (prokaryotic and eukaryotic) was investigated by Corda *et al.* who found that productive elongation was inhibited by both bifunctional and monofunctional adducts. The strongest of these blockages were from 1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpG) intrastrand lesions and the 1,2-d(GpG) interstrand lesion. Conversely, neither the monofunctional *cis*-DDP adducts nor the *trans*-DDP adduct provided effective blockages of transcription.

The primary mode of action was then suggested to be the inhibition of DNA synthesis, the frequency of which extends far beyond the inhibition of RNA or protein synthesis. This hypothesis was supplemented from observations that cells which are deficient in DNA repair mechanisms were more susceptible to *cis*-DDP. In this study, wild-type *E. coli* and mutants lacking the repair function genes *uvrA*, *recB*, *recC* and *polA*, were treated with *cis*-DDP resulting in extensive filamentation in all cases. The viability of the mutants was significantly less than that of the wild-types, suggesting that the damage exerted on DNA by *cis*-DDP was reversible via excision or recombinatorial repair mechanisms. Occurrences of *cis*-DDP resistant cells were reported and these DNA repair mechanisms were suspected to be responsible.

### 1.2.3.2 *cis*-DDP’s Mechanism of Action and DNA Repair

Although both *cis*- and *trans*-DDP form adducts with DNA, the *cis*-isomer has far greater activity. The primary reason suggested for this observation is that *trans*-DDP only forms monofunctional adducts with DNA. These monofunctional adducts are able to react with sulfhydryl groups, such as those of glutathione, which can prevent the formation of a more effective bifunctional adduct. A study by Eastman *et al.* on *cis*-DDP resistant tumor cells showed that, unlike previous reports which suggested resistance could partially be afforded by reduced platinum accumulation within the cell, the major contribution to resistance occurs

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3 Transcription is the enzymatic production of an mRNA copy of a DNA sequence.
after DNA is platinated. Eastman went on to examine DNA repair as a mechanism of resistance to \textit{cis}-DDP by investigating potential differences between sensitive and resistant cell lines.\textsuperscript{58} Accumulation studies showed that resistant cells accumulated 60\% less \textit{cis}-DDP than the sensitive strains although they were able to tolerate drug concentrations 50 times that the sensitive cells could withstand. This clearly reinforced that accumulation contributed little to drug resistance and that other mechanisms were responsible. The removal of adducts via repair mechanisms was also investigated, with resistant cells showing an enhanced and rapid repair of the lesions. The resistant cells removed up to 4 times as many adducts as the sensitive strains during the rapid phase of repair (first 6 h). Despite the efficiency of DNA repair by the various resistant strains in the study, their actual level of resistance did not correlate with their ability to excise and repair the adducts; cells with 100-fold resistance (compared to sensitive strains) were only slightly more effective at repair than those with only 20-fold resistance. It was later discovered that the main DNA repair pathway for these adducts was the nucleotide-excision-repair pathway (NER), but the efficiency of repair was low and did not confer the majority of resistance to the cells.\textsuperscript{59} Conversely, it has been suggested that an increase in DNA repair plays an important role in resistance.\textsuperscript{60} A study by Dabholkar \textit{et al.}\textsuperscript{61} shows that the genes responsible for the NER pathway (\textit{ERCCI} and \textit{XPAC}) in resistant tumor cells have greater levels of the corresponding mRNA transcripts than in those sensitive to chemotherapy.

DNA repair was also seen in other studies to be of major importance,\textsuperscript{62-64} however, all of these analyses were conducted on the ovarian cancer cell line A2780/CP70 which is a low resistance tumor (13-39 fold over baseline). At low resistances it is understandable how DNA repair, proven to be modest in contribution, could be considered an important mechanism of resistance. There is evidence that DNA repair is an early mechanism by which cells respond to the presence of lesions,\textsuperscript{65} with suggestions that it appears to be activated first. At higher levels of resistance the significance of DNA repair is lessened and the majority of resistance requires the induction of other mechanisms.
1.2.3.3 Glutathione Resistance

There is strong evidence that thiol containing peptides are able to confer some resistance to cis-DDP. Glutathione (GSH) is a tripeptide that is highly abundant within cells responsible for protecting against toxins such as free radicals and is also a strong nucleophile, reacting well with alkylating agents. The reaction between GSH and cis-DDP forms a 2:1 GSH-platinum complex, which is exported from the cell via an ATP-dependant glutathione S-conjugate pump. GSH has also been implicated in supporting DNA repair, possibly by enzyme stabilisation or promoting the formation of deoxyribonucleotides. Increased GSH levels are present in some resistant cells, although it has been found that the ability of a cell to synthesise GSH in response to stress is more important than basal GSH levels. Metallothionein (MT) is a protein associated with the detoxification of heavy metal ions from the cell. MT gene transcription is upregulated in the presence of heavy metals and is able to bind up to 10 platinum complexes per molecule. Studies have confirmed that cell lines selected with heavy metals showed increases in MT with the development of cis-DDP cross-resistance.

1.2.3.4 p53 Resistance

One of the more recent theories in the discovery of resistance mechanisms pertains to the signal transduction pathways vital for cell cycle arrest and apoptosis. Of these studies, a large amount of research has been focused toward to the p53 tumor suppressor protein, which has a crucial role in apoptotic processes in response to DNA damage. The importance of p53 sensitivity to cis-DDP has been found to vary in different tissues and cell lines. There is evidence that in some resistant cell lines and tumors where there is a high expression of p53, conversely, there are contradictory reports where p53 signalling sensitises cells. Despite these contradictory results, the most promising study has come from knockout experiments on HCT-116 human colon carcinoma. In this study both alleles of p53 were somatically disrupted which resulted in hypersensitivity to cis-DDP, believed to be the result of failure to induce cyclin-dependant kinase inhibitor p21 expression. p21 is the primary mediator of p53 induced cell cycle arrest and has been determined nonessential for p53-
dependant apoptosis in thymus\textsuperscript{76} and intestinal crypts\textsuperscript{77} of p21-deficient animals and was also not required for oncogene-induced apoptosis.\textsuperscript{78} The belief that loss of p53 function results in cis-DDP hypersensitivity has been supplemented by studies on A2780 cell lines where p53-deficiency results in the loss of G1/S checkpoint\textsuperscript{**} control and adduct repair when compared to A2780 cells with functional p53.\textsuperscript{79} Despite some optimistic results, the effect of p53 on cis-DDP sensitivity varies with cell type and its exact role in resistance remains ambiguous.

1.2.3.5 Cell-Adhesion Mediated-Drug Resistance

In recent years, several new ideas have emerged in the field of platinum resistance. Studies concerning the tumor microenvironment have highlighted some important issues. There is evidence that cell-cell contacts, in particular E-cadherin interaction, can effect drug resistance.\textsuperscript{80} Cadherins are an important class of proteins responsible for inter-cellular adhesion, where E-cadherins are those proteins found on epithelial cells. This type of drug resistance is known as cell-adhesion mediated-drug resistance (CAM-DR). It was found that ovarian tumors often overexpressed collagen VI and that cells grown in such an environment show resistance to cis-DDP.\textsuperscript{81} This suggests that resistant cells may be able to rearrange their environment to better their interaction with the extracellular matrix and decrease their susceptibility to apoptosis. Unfortunately, contradictory evidence has been found concerning the importance of E-cadherin interactions and platinum resistance,\textsuperscript{82, 83} signaling the requirement for further investigation into this novel aspect of drug resistance. It has been generally been thought that cis-DDP toxicity is autonomous with each case a result of the formation of DNA adducts. Recent studies show that toxicity can be transduced into neighbouring cells via gap junctions; a nexus between certain cell-types composed of two connexon proteins where molecules and ions are allowed to pass between the cells.\textsuperscript{84} While studying the effect of DNA repair complex Ku70/80 on resistance, it was discovered that cells plated at high density were sensitive while the same cells plated at low density were not as susceptible. Investigations found that damage caused by cis-DDP can activate DNA-\textsuperscript{**}The G1/S checkpoint is composed of a series of safeguards between G1, the major growth phase, and the S phase, were cellular division begins. This checkpoint ensures there is no damage to DNA and cellular function is normal.
dependant protein kinase (DNA-PK) to initiate a death signal that can be transmitted from
cell to cell through gap junctions. This could lead to strategies that can manipulate gap
junctions, hopefully improving the efficacy of existing platinum drugs. Unfortunately, the
loss of E-cadherin interactions has also been implicated in the progression of cancer. The loss
of cellular adhesion enables cells to be motile, consequently, cancerous cells are able to
invade surrounding tissues.

1.2.3.6 MUC1 Resistance

In epithelial cancers cis-DDP resistance can be linked to the over expression of MUC1 (an
integral membrane glycoprotein). It has been found that the C-terminal subunit of MUC1
localises to the mitochondria in various cancers. It is this localisation that leads to blockages
of the intrinsic apoptosis pathway in these cells. This is thought to arise through the decrease
in cytochrome c release from the mitochondria and reduced activation of capase-3, both
proteins which are necessary for apoptosis. To solidify these findings, it was shown that
down regulation of MUC1 expression in cells that were overexpressing the protein lead to
sensitisation. Thus, strategies targeting the inhibition of MUC1 expression or localisation to
the mitochondria could present a solution to combat some resistances to platinum drugs.

1.2.3.7 The Future of cis-DDP Resistance

It is evident that cis-DDP resistance has attracted a significant amount of research from
investigators and while the elucidation of these resistance mechanisms remains and important
area of study, the reversal of these resistance pathways is equally paramount. In doing so cis-
DDP can be used with the full efficacy of the drug restored. Approaches to overcome
resistance have concentrated on developments of cis-DDP analogues, in a hope that
resistance-patterns are not shared between drugs that act via different mechanisms. This
appears to be a sound strategy, although in reality cross-resistance is commonly observed in
tumor cells. This problem is most likely due to the fact that resistant cells are less prone to
apoptosis, a concern that may be independent from drug type. Nevertheless the
identification of new tumor targeting cytotoxic agents remains an important avenue of
research to combat resistance. The underlying mechanism of platinum resistance in tumor cells remains to be definitively answered, despite the great advances in current research.

1.2.3.8 trans-Platinum Complexes

Since the discovery of diamminedichloroplatinum(II), the cis-isomer was identified as the active form whilst the trans-isomer was found to be ineffective at arresting *E. coli* cell division.\(^{44}\) This lead to the consensus that *trans* geometry was not active. To be considered active an analogue must have: (1) *cis* leaving groups (2) be uncharged (3) leaving groups that are moderately bound (compounds with highly labile leaving groups are toxic while those that are too tightly bound are less active).\(^{87}\) Despite these requirements, physio-chemical studies of *trans*-DDP have shown that it is more reactive than *cis*-DDP. *trans*-DDP will react quickly with nucleophiles in the dichloro and chloro-aqua species whereas the diaqua form is relatively slow.\(^{87}\) Consequently, *trans*-DDP is more susceptible to undesired side reactions such as GSH binding, before reaching its pharmacological target, which could in part explain its lack of anticancer activity. It was suggested by Farrell that the antitumor activity of *trans* platinum complexes could be improved by use of bulky carrier ligands which would reduce the rate of chloride exchange.\(^{88}\) The steric environment created by these ligands would also limit access to the platinum center and inhibit the formation of the five-coordinate intermediate prior to the chloride displacement. To examine this theory, three classes of complexes were investigated of the type *trans*-[PtCl\(_2\)(L)(L')] (Figure 1.11).\(^{89}\)

The cytotoxicity of all these analogues was greater than that of *trans*-DDP by at least one order of magnitude and were also as cytotoxic as their *cis* counterparts.\(^{89}\) Although the structures of these analogues varied, they shared similar cytotoxicity profiles towards tumor cells resembling the antibiotic rifamycin (mechanism of action via inhibition of DNA-dependant RNA polymerases) rather than *cis*-DDP.
A general trend in these complexes showed that bulky planar ligands did not share cross-resistance with cis-DDP in murine L1210 leukaemia and human ovarian tumor cells with cis-DDP resistance. The bipyridine analogues (Figure 1.11) appeared to have greater cellular uptake compared to trans-DDP (L1210) and were also found to inhibit DNA synthesis. The binding of the pyridine complexes to calf thymus DNA (ct-DNA) is significantly less than that of trans-DDP. The presence of the bulky pyridine ligands creates steric conflicts that weaken DNA binding as well as binding to other biomolecules such as GSH. Although binding affinity is reduced, the lowered reactivity offers some degree of protection from miscellaneous binding as the complex travels to it’s DNA target.

Replacement of a single NH$_3$ with an aromatic amine is found to dramatically increase the cytotoxicity of trans analogues. [PtCl$_2$(NH$_3$)(thiazole)] (Figure 1.11) shows in vivo antileukaemic activity which is not seen in the trans-bispyridine complexes. Binding studies of [PtCl$_2$(NH$_3$)(quinoline)] have shown that monofunctional adducts coordinate to DNA at a rate similar to trans-DDP. The rearrangement to bifunctional adducts was also reported to be similar to trans-DDP; after incubation in ct-DNA for 48 h, 34% of the total adducts

Figure 1.11 Structures of active trans-platinum complexes, a L = L’ = pyridine, N-methylimidazole or thiazole, b L = quinoline and L’ = MeRSO (where R = Me, Bz or Ph) and c L = NH$_3$ and L’ = quinoline and thiazole.
formed remained monofunctional. Both the quinoline and thiazole complexes have an interstrand cross-linking efficiency (up to 30%) greater than *cis*-DDP (6%) or *trans*-DDP (12%). The conformational changes to DNA caused by [PtCl₂(NH₃)(thiazole)] or [PtCl₂(NH₃)(quinoline)] are quite different from those produced by *trans*-DDP, but instead resemble the 1,2-intrastrand (GG) cross-links created by *cis*-DDP. Molecular modelling showed that these structural distortions are a combination of the monofunctional platination and the quinoline group partially intercalating into the DNA bases. It is believed that the interstrand adducts formed may contribute to the antitumor efficacy of these complexes.

In addition to the more traditional types of *trans* platinum complexes, various analogues have been made that are highly active. The most recognised of these is Farrell’s BBR3464 (Figure 1.12). This novel polynuclear complex is composed of three platinum centers linked by hexanediamine chains. Phase I trials of BBR3464 demonstrated a pattern of responses in cancers not usually treatable with *cis*-DDP while phase II trials demonstrated it has 100 times the cytotoxicity than *cis*-DDP. It is believed the 4+ charge of the complex contributes extensively to its anticancer profile with its mechanism of action different to that of *cis*-DDP.¹⁹¹

The binding of BBR3464 to DNA is characterised by the rapid formation of long range intra- and interstrand crosslinks. Binding studies conducted on the palindromic octamer 5'-d(ATGTACAT)_2-3' found that coordination occurred at guanine residues four base pairs apart. The most interesting feature of its binding though, is the lack of structure distortion such as the kink seen in *cis*-DDP binding, which suggests it is not recognised by proteins responsible for NER (DNA repair). It has been reported that the 1,4-interstrand cross-links are not removed by NER and are able to persist longer within the cell than other platinum drugs. Conversely, 1,4-intrastrand cross-links create localised distortions that are susceptible to NER and that processing of these lesions do not impact on its antitumor effects. This implies a significantly different mode of activity and coincides well with the compounds ability to overcome *cis*-DDP resistant tumors.⁹⁴
1.3 Sequence Selective Polyamides

Thriving within the earth’s soil, *streptomyces* is a genus of microscopic organisms that have contributed significantly to modern day medicine. With their filamentous mycelium that resemble fungi in structure, they secrete extracellular hydrolytic enzymes to obtain nutrients from their surroundings. They are able to metabolise a variety of compounds including amino acids, alcohols and aromatic compounds, consequently making them important contributors in the degradation of organic matter. Despite their vital niche in the world’s ecology, *streptomyces* most significant characteristic is their production of over half the known antibiotics in existence. Amongst the many of antibiotics secreted by these bacteria, are Distamycin (Dst) and Netropsin (Nt), (Figure 1.13) two natural products that have paved the future of sequence selective polyamides.

Dst and Nt were first discovered by Finlay\textsuperscript{95} et al. and Arcamone\textsuperscript{96} et al., during the advent of molecular research in the 1950s. It was a time during which the structure of DNA was about to be solved\textsuperscript{1} and the surge of structural investigations into DNA were being supplemented with studies concerning the interaction of DNA with various ligands. DNA binding analyses of these drugs were not conducted until almost two decades after their initial discovery.\textsuperscript{97-99} The compounds were found to have interesting characteristics as a result of their unique binding. It was discovered the compounds had an A-T binding preference\textsuperscript{100, 101} and, intriguingly, the pathogens most effected were A-T-rich organisms. Despite the successes *in vitro*, Dst could not be commercialized due to problems concerning
cellular uptake and solubility. Regardless, the foundations of sequence specific technologies were laid, and the enormous potential of this class of compounds have continued to demand further investigation.

1.3.1 Interactions of Distamycin and Netropsin with DNA

The DNA binding abilities of Dst and Nt (Figure 1.13) were first discovered by Zimmer et al.\textsuperscript{97} and the findings were largely based upon solution data. Circular dichroism (CD) based binding studies of these compounds showed a preference for A·T rich sequences, while weak or no binding was observed for G·C base pairs.\textsuperscript{100, 101} It was reasoned this specificity was due to hydrogen bonding between carboxamides with the O(2) atoms of thymines and the N(3) of adenine bases.\textsuperscript{102-104}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.13}
\caption{Structures of a Distamycin-A and b Netropsin, consist of pyrrole rings linked via amide bonds, possessing a head (N-terminus), and a tail (C-terminus).}
\end{figure}
This theory was reinforced by binding experiments conducted on a Dst analogue which had the amide groups replaced with methylamides, whereby no binding was observed for the analogue in the presence poly(dA)-poly(dT).\textsuperscript{105, 106} Kopka \textit{et al.} proposed that hydrogen bonds are not directly responsible for the specificity of the compounds, rather, they serve to stabilise the binding to the floor of the minor groove.\textsuperscript{107, 108} Instead, the specificity to dA·dT pairs is a result of van der Waals interactions between C-H groups on the pyrrole (Py) rings and C(2) hydrogens on adenine. This sequence-specificity for A·T bases can also be viewed as the compounds inability to bind to C·G bases; the 2-amino group of guanine residues sterically hinder drug binding as evidenced by Nt-dodecamer complex crystal structures obtained by Kopka \textit{et al.}\textsuperscript{107-109}

Initial NMR studies conducted by Patel,\textsuperscript{110} showed Nt bound to the minor groove of DNA. Subsequent NMR and crystallographic studies on Nt\textsuperscript{107, 108, 111} and Dst\textsuperscript{112-114} showed a more detailed picture of their binding. These investigations saw that their crescent shapes complemented the curvature of DNA well, and that the amide protons pointed into the groove. Interestingly, early affinity cleavage studies and subsequent NMR experiments showed that the binding of Dst to heterogeneous DNA sequences was orientation specific; the N-terminus favoring the 5‘ end.\textsuperscript{115-117} The positively charged tail of Dst forces an orientation with DNA to produce the most energetically favourable binding. Considering the structure of Nt, it is not surprising that there was no specificity with its orientation as both head and tail share very similar characteristics as seen in Figure 1.13.

### 1.3.2 Analogues of Dystamycin and Netropsin

Initial modifications of the naturally occurring compounds were aimed at increasing the number of 1-methylpyrrolecarboxamido residues. It was found that by progressively increasing the residues from 3(Dst-A or Dst-3) to 6(Dst-6), a corresponding increase in melting temperature and changes in UV absorbances with dsDNA was observed.\textsuperscript{118} This increase in affinity from Dst-3 to Dst-6 was believed to be a result of increased number of interactions between DNA and the analogue.
Subsequent modifications to Nt involved covalently linked Nt-like molecules.\textsuperscript{119} These bis-Nt molecules, consisting of head-to-head, tail-to-tail and parallel arrangements, all showed increased binding affinity to poly(dA)·poly(dT) compared to monomeric Nt.\textsuperscript{120} It was determined with CD studies that the linker length had an impact on binding affinity; a 6-carbon chain linker provided stronger binding than a 2-carbon chain. It is believed that the longer linker enables independent binding of both Nt-like molecules to the dimeric compound, while the shorter linker restricts movement and binding of the both Nt molecules. In 1983, Schultz and Dervan\textsuperscript{121-123} synthesised Dst-A and Dst-5 molecules tethered to the DNA cleaving agent ethylenediaminetetraacetic acid (EDTA)-Fe(II). This attachment provided the ability to monitor the base pair span of their binding sites. The Dst-A binding site covered 5 bp, while Dst-5 spanned 6 to 7 bp.

At the same time, the groups of Dickerson\textsuperscript{108} and Lown\textsuperscript{124} suggested that the substitution of a pyrrole ring for an imidazole, labeled at the time as “Lexitropsins” might confer the ability to code G·C. The idea was that by removing the C-H(4) and replacing it with a nitrogen would now permit a hydrogen bond and remove the steric conflicts. The first attempt made involved a simple molecule (Figure 1.14) consisting of a pyrrole coupled to an imidazole ring.\textsuperscript{125} The NMR study conducted by Lown proved that there was a greater affinity for G·C sequences, unfortunately, the compound did also bind to A·T sequences.

![Figure 1.14](image-url)  Attempting to solve the G·C code; the first Lexitropsin produced by the Lown group.
Meanwhile, Dervan’s group was also trying to overcome the G·C recognition problem. Following Lown’s suggestion for the need of a hydrogen bond acceptor, Dervan substituted a pyrrole ring with a pyridine. The binding of the analogue (2-PyN) was monitored by MPE·Fe(II) footprinting and an affinity for G·C sequences was seen, although it raised some questions which at the time could not be answered. A strong binding to 5'-GTC-3' was also reported which was not expected as the compound should have bound 5'-GTA-3'. It was then believed that somehow the terminal pyrrole carboxamide was hydrogen bonding with the NH$_2$ of guanine and that the original model needed to be revised (Figure 1.15).

An important discovery while studying Dst binding to A·T sequences was made by Wemmer et al. NMR experiments showed that distamycin was not only binding in 1:1 ratio to DNA, but also manifested as 2:1. The most obvious difference noted in 2:1 binding was the substantially wider groove not normally seen in A-T rich sequences alone, although, it was debated whether 2 minor groove binders could fit side-by-side. This dimeric binding had the same characteristics as the binding observed for 1:1 stoichiometries; the contacts with the minor groove floor were present, along with C-H interactions with the bases. Titrations of the AAATT sequence with distamycin showed at low concentrations the 1:1 complexes formed, while at high stoichiometries the 2:1 complex was dominant. A feature of the 2:1 binding was that the molecules completely overlapped and that they did so running antiparallel; the tail of one contacted the head of the other.

With the landmark discovery by Wemmer, Dervan was given the missing information. His compound was binding antiparallel as a 2:1 complex. This work was not published until 1992, when Dervan collaborated with Wemmer to prove by NMR that the 2:1 mechanism solves the G·C recognition problem. It was uncovered that when an imidazole from one polyamide overlapped a pyrrole from another in a 2:1 complex with DNA, it specifically recognised G·C.
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Figure 1.15 Model for binding of 2-PyN to 5'-GTC-3'. The dashed lines are the proposed hydrogen bonds formed. Circles with two dots represent lone pairs of electrons on N(3) adenine and O(2) of cytidine and thymine. Circles containing an H represent N(2) proton of guanine.

A study concerning the maximum number of rings coupled sequentially was investigated by Kelly et al.\textsuperscript{129} The binding of six polyamides, containing between three to eight rings each, have been shown to span five to ten base pairs respectively. Sequence specificity was seen to decrease when the polyamides exceeded five rings. This anomaly was likely due to the changing curvature of the polyamide, which, beyond five rings fails to match the torsional constraints of the DNA helix. This would lead to fewer interactions between hydrogen bond donators and acceptors. As a result, the unfavorable interactions between the polyamides and DNA which maintain specificity are effectively lost reducing differences between match and mismatch sites. With these results showing a maximum affinity reached at five rings, a new class of compounds was required to maintain selectivity when targeting sequences greater than 8 base pairs. This could be achieved via insertion of a flexible linker every four or five rings or by simply changing the structural design of these molecules.
1.3.3 Hairpin Polyamides

After solving G·C recognition, Dervan attempted to address the problem that exists when two different peptides bind as a heterodimer. When heterodimerisation does occur there is the inherent ability for each peptide to also bind as a 1:1 complex instead and thus offer multiple binding sites. In order to address this problem, the peptides were covalently linked by butyl linkers through central pyrrole rings. The first generation covalent peptide dimers had increased affinity and specificity, but the methodology to construct these molecules could not be easily adapted to prepare other peptide analogs.

The second generation covalent peptide dimers utilised flexible carbon chain linkers, designated ‘hairpins’. These hairpin polyamides afforded a more general synthetic procedure that can be applied to various types of analogs. Linked in a head to tail arrangement, the linear peptide molecule folds back upon itself to create the antiparallel structure. It therefore becomes apparent that the linker length between the two peptide strands would greatly affect the ability to form this conformation. Mrksich et al. were able to confirm this hypothesis by testing the binding of a series of hairpin peptides (Figure 1.16).

![Figure 1.16](image_url)

**Figure 1.16** The hexapeptides made by Mrksich et al. where n = 1 (2-ImN-GLY-P3); n = 2 (2-ImN-βALA-P3); n = 3 (2-ImN-γABA-P3); n = 4 (2-ImN-AVA-P3).
The linkers used in the investigation were glycine (gly), β-alanine (β-ala), γ-aminobutyric acid (γ-ABA) and aminovaleric acid (AVA), with respective carbon chain lengths of 1, 2, 3, and 4. Of the four hairpin polyamides, 2-ImN-γABA-P3 bound to the target sequence 5'-TGTATA-3' with the highest affinity and sequence specificity. Also, the same peptide showed a 24-fold lower affinity to the mismatch sequence 5'-TGACA-3'. This indicates the γ-aminobutyric acid linker promotes a turn in the peptide and thus a 2:1 (intramolecular) binding mechanism rather than an extended conformation, which produces 1:1 or 2:1 (intermolecular) binding.

1.3.4 A·T / T·A Specificity

While the interactions between an Im/Py combination and a G·C pair are strand-specific, the recognition of A·T/T·A pairs by Py/Py are degenerate; there was no specificity for A or T bases. A Py/Im pair is able to distinguish energetically between G·C and C·G because the adjacent Py pushes an Im to the side of the minor groove resulting in hydrogen bond formation between N(2) of guanine and N(3) of imidazole. The specificity observed with the singular change between Py and Im at position 3 indicated that it might be possible to apply this approach to a pyrrole ring in a Py/Py pair to discriminate between A and T bases. The difficulty in this approach was the similar positioning of the hydrogen-bond acceptors, N(3) of adenine and O(2) of thymine, within the minor groove.132

Expanding upon principles of imidazole specificity, Dervan and coworkers group introduced a new aromatic ring, 3-hydroxypyrrole (Hp), to the recognition code.133 The selectivity achieved could arise via stabilisation, hydrogen bonding with the hydroxyl group of Hp and the carbonyl of thymine, or via destabilisation, where the hydroxyl group of Hp causes steric conflicts with the hydrogen bond donators of adenine. With the addition of Hp to the repertoire of sequence specific rings, it was now possible to specifically target the four Watson-Crick base pairs using synthetic polyamides.
1.3.5 Gene Regulation with Polyamides

The primary function of DNA is the storage of hereditary information in units known as genes. There are approximately 35,000-50,000 genes present within each human cell, each consisting of a sequence of DNA that occupies a specific location on a chromosome and is responsible for the phenotype of a particular organism. With the development of polyamides that are able to recognise specific base pair sequences, it became possible to target a specific gene. By selectively targeting a gene sequence, it is feasible that gene expression levels could be altered providing an invaluable tool that can be used to create novel DNA binding drugs.

1.3.5.1 Transcriptional Inhibition

The expression of a gene is controlled by an array of protein complexes known as transcription factors. These transcription factors assemble with RNA polymerase on highly specific sequences of DNA in the promoter region of each gene, which spans several hundred base pairs. Once these factors are assembled transcription may commence, producing an mRNA strand resulting in gene expression. If the binding of transcription factors is inhibited gene expression does not proceed. It is known that the inhibition of transcription is prominent in over half the anticancer agents in current clinical use. These drugs act by inhibiting the binding of transcription factors to DNA, therefore preventing initiation, or by creating drug-DNA adducts, hindering the migration of RNA polymerase downstream during elongation.

A study was conducted by Dervan and coworkers to determine if a synthetic hairpin polyamide could inhibit transcription. The eight-ring polyamide, ImPyPyPy-γ-ImPyPyPy-β, was used to target the recognition site of the transcription factor TFIIIA and was monitored both in vivo and in vitro. Both results showed that transcription of 5S RNA genes in Xenopus kidney cells were inhibited. The results also showed that the pyrrole/imidazole polyamides are cell membrane permeable. A second study was then conducted on the human immunodeficiency virus-1 (HIV-1) enhancer/promoter elements containing multiple transcription factor binding sites, including TBP, Ets-1 and LEF-1. The DNA binding of
these transcription factors were inhibited by two hairpin polyamides that targeted adjacent sequences as well as inhibiting HIV-1 transcription in vitro. Tests on human peripheral blood lymphocytes using these two polyamides showed that, synergistically, virus replication was suppressed by more than 99% with no apparent cellular toxicity.\textsuperscript{36}

Sugiyama and coworkers also investigated transcriptional inhibition using hairpin polyamides linked to the alkylating agent cyclopropapyrrloindole (CPI). It has been shown that hairpin polyamide-CPI conjugates are able to efficiently alkylate double-stranded DNA at a predetermined position.\textsuperscript{137} Further investigation revealed that the alkylating polyamide, ImPyPy-\textgamma-ImPyLDu86, effectively inhibited transcription. This was confirmed to occur precisely at the site of alkylation resulting in the formation of truncated mRNA transcripts.\textsuperscript{138}

Sugiyama then tested sequence specific gene silencing in mammalian cells with alkylating polyamides.\textsuperscript{139} In this study, two alkylating agents were investigated, ImImPyPy-\textgamma-ImImPyLDu86 and ImImPyPy-\textgamma-ImPyPyLDu86, which targeted coding regions of renilla luciferase and firefly luciferase (enzymes which catalyse light producing reactions) respectively. Two plasmid vectors encoding renilla luciferase and firefly luciferase were alkylated using the polyamides and transfected into HeLa, 293 and NIH3T3 cells. Luciferase expression was then assayed and showed that gene silencing was effective in all cases. The vectors were then cotransfected into HeLa cells and then treated with the alkylating polyamides. Results showed a reduction in both luciferase activities, proving they have potential in vivo as novel drugs capable of regulating gene expression.

\textbf{1.3.5.2 Transcriptional Activation}

The data supporting transcriptional inhibition by sequence selective polyamides raised the question; is it possible to activate transcription using artificial groove binders? Positive regulation of transcription requires a group of proteins called activators. Activators facilitate transcription by binding to DNA and recruiting the RNA polymerase II holoenzyme (pol II holoenzyme), a complex containing multiple protein subunits that associate with RNA
polymerase II, to the promoter region. PolII holoenzyme contains a domain that binds to enhancer elements and an activation domain, both of which stimulate transcription of the particular gene.

Using the knowledge of recruitment††, attempts have been made to create artificial transcriptional activators. Mapp et al. designed a synthetic activator, where the DNA binding domain of the protein was replaced with a hairpin polyamide. Also included in the molecule was a dimerisation domain, as it was unclear whether this module was necessary for the activation of the gene, and the activation domain AH (Amphipathic Helix). Results showed that this molecule activated transcription in vitro in yeast nuclear extract on a template containing a palindromic binding site targeted by the hairpin polyamide. In the control molecule lacking the dimerisation domain, transcription still took place, proving it was not necessary for activator function. Removal of the activation domain resulted in no activation of transcription. Expanding upon these results, Dervan investigated the effect of linker length between these hairpin polyamides and their activation domains. Looking at the principle of recruitment, the activation domain must have sufficient spatial distance to recruit transcriptional machinery. Exceeding a certain distance from the activation domain will hinder the recruitment of machinery to the promoter. It was found, through in vitro studies, that a spacing of 36-45 Å is the optimal distance between the DNA binding module and activation domains to promote transcriptional activation.

An investigation of artificial regulators based upon the Homeobox (Hox) family of proteins was conducted by Ansari et al. Hox family proteins contain a homeodomain; a trihelical DNA-binding domain that is highly conserved. The role of these proteins is to control the development of anterior and posterior patterning of an embryo. Hox proteins usually display low DNA binding affinity but when they heterodimerise with TALE (Three Amino Acid Loop Extension) class of Hox proteins they bind with a much higher affinity and selectivity. These two types of Hox proteins interact via a short docking peptide (YPWM) that is bound

†† Recruitment: the directed movement of a protein to a specific location.
to the N-terminus of the Homeobox via a variable linker. Substitution of the Homeobox protein with a hairpin polyamide linked to an YPWM peptide recognition site has interesting consequences. The binding of the YPWM polyamide conjugate to DNA results in a bidentate binding site (the DNA binding site and the peptide) that successfully recruits TALE transcription factor. Surprisingly, this recruitment of the TALE protein using the polyamide conjugate is done more effectively than the natural Hox counterpart.

1.4.2.1 Limitations of Gene Regulation

The ability to regulate gene expression through the use of artificial transcription factors presents many possible applications but not without some complications. Trials of polyamides in vitro have showed promising results, but their ability to work within biological systems requires revision upon the design of some of these compounds.

The entirely synthetic polyamide STF1 (synthetic transcription factor 1) is an eight-ring hairpin polyamide coupled to the artificial activator, Wrenchnolol, a wrench shaped synthetic compound that binds to the Sur-2 protein. The Sur-2 protein, a subunit of human mediator complex, is responsible for binding transcriptional activators to RNA polymerase II.\textsuperscript{143, 144}

The effectiveness of STF1 to activate transcription in vitro was monitored by the use of a reporter gene in a study conducted by Uesugi et al.\textsuperscript{145} Results showed that STF1 activation of the reporter gene was successful, while the control, which lacked the wrenchnolol moiety, showed no activity. Progressing to in vivo studies, the cellular uptake of STF1 was monitored using the fluorescein\textsuperscript{14} labeled form of the compound. Unfortunately, STF1 was not cell permeable, although all of the individual components of the molecule had cell permeability. Problems concerning cellular uptake are well known, with restricted cell permeability being a result of, but not limited to, molecular weight. The complications in gene regulation are not restricted to artificial activation of in vivo systems. The HER2/neu oncogene is up-regulated transcriptionally in 25-30% of human breast cancers.\textsuperscript{146}

\textsuperscript{14} Fluorescein is a fluorophore commonly used in fluorescence microscopy to track cells or labelled molecules within cells.
Site (EBS), a highly conserved sequence of the HER2/neu oncogene promoter, and inhibit transcription with select hairpin polyamides have proven to be successful. \textsuperscript{146} In vitro studies confirmed that these polyamides effectively inhibit HER2/neu promoter-driven transcription using nuclear extract from the human breast cancer cell line, SKBR-3. In vivo tests against this cell line were ineffective.

Several major concerns exist when examining the requirements for \textit{in vivo} gene regulation. Firstly, there are cellular uptake and trafficking issues. A DNA binding molecule must not only transverse the cellular membrane, but navigate past the nuclear membrane to reach its target. Eukaryotic DNA is stored within the nucleus in a highly compacted state known as chromatin. Chromatin achieves its densely folded structure by wrapping around an assembly of disk-shaped proteins called histones. Eight histone-DNA complexes, which can be described as beads on a string, are termed nucleosomes which not only serve to compact DNA, but are also present to affect accessibility of molecular machinery such as DNA binding proteins. It has been found that nucleosomes can also affect the binding of polyamides to DNA. \textsuperscript{147} Sites that are completely blocked are those where there are interactions between histones and DNA, while those that are facing away from the histones are fully accessible.

\textbf{1.5 Basis for This Project}

\textbf{1.5.1 Aim of This Study}

This project was undertaken to develop polyamides with the added functionality of metal complex conjugates. Attachment of ruthenium(II) and platinum(II) complexes is proposed to enhance the binding of polyamides to DNA by: (1) increasing DNA affinity (increased positive charge); (2) attachment of complexes capable of intercalation will increase DNA stabilisation, which groove binders are generally incapable of; (3) attachment of complexes that bind coordinatively to DNA will give the compound an irreversible mode of binding and afford increased functionality to the polyamide that is seen with successful drugs such as
cisplatin. The metallo-polyamides investigated in this project, LLSP4-Pt and LLSP4-Ru are shown in Figure 1.17.

Figure 1.17  The metallo-polyamides a LLSP4-Pt and b LLSP4-Ru.

1.5.2 Outline of Thesis

This thesis is an account of the synthetic process, DNA binding studies and biological testing of a number of novel metallo-polyamides types, including both platinum(II) and ruthenium(II) conjugates.

CHAPTER 2. The synthetic approaches in the development of select polyamides and the platinum(II) linked metal complex LLSP4-Pt are detailed here. Experimental details of synthesis and characterisation are provided, with an account given on the limitations of solution phase chemistry found when synthesising these compounds and the advancement to solid phase supports.

CHAPTER 3. The development of the ruthenium/polyamide conjugate LLSP4-Ru is described. Experimental details of various ligand and metal complex syntheses and characterisations are detailed. In addition, the synthesis of the target polyamide LLSP4-Ru via solid phase methods is described using techniques modified from Chapter 2.

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The polyamide names are abbreviations of ‘Linear Linker Solid Phase 4 ring’ polyamide.
CHAPTER 4. The binding of LLSP4-Pt to DNA was investigated using several techniques. Binding of LLSP4-Pt to guanosine was monitored by NMR in order to monitor coordination of platinum to the N7 of guanine. The binding of LLSP4-Pt to ct-DNA was monitored using circular dichroism, where the reversible binding at high salt concentrations was measured (200 mM). Using the CD data, a binding constant was determined for the metal complex.

CHAPTER 5. A summary of the results and research described in this thesis. Here conclusions that were drawn throughout the study are pooled and reviewed as a whole. Discussions of synthetic strategies and suggestions for future method refinements are reviewed in addition to the directions of future research in the field of metallo-polyamides.
CHAPTER 2: POLYAMIDE SYNTHESIS

2.1 Introduction

The synthesis of peptides via solution phase chemistry has been explored extensively, dating as far back as 1902.\textsuperscript{148} The focus of peptide chemists throughout the 20\textsuperscript{th} century had been the total chemical synthesis of natural peptides, which has expanded not only the synthetic routes to peptides but also the synthesis of peptide based compounds such as pyrrole/imidazole polyamides. Previous methodologies of polyamide synthesis have been dependent on solution based coupling reactions and have been used to create a vast number of compounds structurally similar to Distamycin (Dst) and Netropsin (Nt).\textsuperscript{149-154} Despite the success of these methods, advances in peptide chemistry have seen the development of solid phase techniques that produce polyamides with higher purity and faster coupling times.\textsuperscript{155, 156}

Metal complexes that are able to bind to and modify nucleic acids have received great interest as potential therapeutic agents or DNA probes.\textsuperscript{157-159} The attachment of transition metal complexes to polyamides has been explored with the attention of this research focusing on the development of sequence specific artificial nucleases using DNA cleaving agents such as Fe(II)-EDTA and [Cu(phen)\textsubscript{2}]\textsuperscript{2+}.\textsuperscript{160, 161} Additionally, polyamides have been investigated as carriers of antitumor drugs such as cisplatin (\textit{cis}-DDP).\textsuperscript{162, 163} The two isomers of the chiral compound [Pt-DIST], a conjugate of \textit{cis}-DDP and Dst, formed higher percentages of interstrand cross-links with DNA, compared to natural \textit{cis}-DDP. Its activity was attributed to the binding of the distamycin ligand in the DNA minor groove which caused distortions of the helix. \textit{cis}-DDP’s specificity for G·G or A·G sequences did not change despite the attachment of the DNA sequence specific Dst group, and the complex formed intrastrand DNA adducts as natural \textit{cis}-DDP does.

A study from the Aldrich-Wright group on \textit{trans}-DDP/polyamide conjugates has provided the structural basis for agents that are able to bind specific sequences of DNA and inhibit DNA transcription and replication.\textsuperscript{164} The two compounds investigated were DJ1953-6 and
DJ1953-2 (Figure 2.1) which have a DNA target sequence of 5'-TGTCA-3', and differed only by the carbon linker length between the platinum group and the three ring polyamide. The study reports DJ1953-2 is able to unwind the DNA helix by 13° but neither metal complex significantly affects the melting temperature of DNA. Although the complexes showed poor activity against the L1210 murine leukaemia cancer cell line (DJ1953-6, IC_{50} = 34 μM; DJ1953-2, IC_{50} = >50 μM), it was shown that the latter could inhibit DNA transcription \textit{in vitro}. This is an uncommon feature as most mononuclear platinum complexes and simple polyamides are incapable of terminating the migration of RNA polymerase during transcription.

![Chemical structures of DJ1953-6 (a) and DJ1953-2 (b).](image)

**Figure 2.1** The platinum polyamide conjugates a DJ1953-6 and b DJ1953-2.

In the present study a new platinum(II) polyamide, LLSP4-Pt (Figure 2.2) has been designed and made by solid phase techniques. LLSP4-Pt is a 4 ring, pyrrole-based polyamide with two \textit{trans}-DDP complexes tethered to the tail of the molecule. As with [Pt-DIST], it is hypothesised this compound will bind to DNA via the minor groove but it remains unknown whether the G·C specificity for the monofunctional adducts will be affected by the A/T DNA sequence specificity of the polyamide. The theoretical binding site of this polyamide will be 'WWWWS' (NC-IUB nomenclature).^{165***} This chapter reports the development of the polyamide LLSP4-Pt from solution methods and initial solid phase attempts (WANG resin) to success with chlorotrityl resin and characterisation by various methods.

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*** The Nomenclature Committee of the International Union of Biochemistry recommends all ambiguous bases are abbreviated. In this case W = A or T, S = G or C.
Figure 2.2  Chemical structure of the platinum polyamide conjugate LLSP4-Pt.

2.2  Reagents

Triethylamine (TEA) 99.5%, tetrahydrofuran (THF), acetic anhydride (Ac₂O) 99.5%, nitric acid (HNO₃) >69%, dimethylformamide anhydrous, tert-butyl alcohol 99.5% high performance liquid chromatography (HPLC) grade, titanium(IV) tetrachloride 1 M in dichloromethane (DCM), aluminium oxide (activated basic, Brockmann I, standard grade ~150 mesh, 58 Å) and N,N-diisopropylethylamine (DIEA) (redistilled 99.5%), piperidine 99.5+% biotech grade were all purchased from Sigma-Aldrich. N-hydroxybenzotriazole (HOBt), Fmoc-β-Alanine-OH-WANG resin 0.70 mmole/g, trifluoroacetic acid (peptide synthesis grade) and 2-chlorotrityl chloride resin (1.0 mmol/g) were all purchased from Auspep. N-Methylpyrrole, 99% and sodium tert-butoxide 97% were purchased from Alfa Aesar. O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), Fmoc-Cl, 98.7% purity and 9-fluorenylmethyl chloroformate (Fmoc-Cl) 98.7% were purchased from Bachem. Trichloroacetic acid (AR grade) was purchased from Chem-supply. N,N'-Dimethylformamide (DMF) (puriss ≥99.8%), N-methyl-pyrrolidinone (NMP), Fmoc-β-alanine (puriss >99%) and 4-(Fmoc-amino)butyric acid (p rumor ≥97%) were purchased from Fluka. 1-Methylpyrrole-2-carboxylic acid (98%) was purchased from Lancaster. Thin Layer Chromatography (TLC) (aluminium sheets, silica gel 60 F₂₅₄), thionyl chloride (synthesis grade) and silica gel 60 were purchased from Merck. O-Benzotriazole-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU) was purchased from Protein technologies. Silica gel (ultra pure, 230-400 mesh) was purchased from Silicycle. 4-(Fmoc-amino)-1-methylpyrrole-
2-carboxylic acid (Fmoc-py-COOH) was obtained from the pyrrole monomer synthesis section (see Section 2.5). All other reagents were of general laboratory grade and used without further purification, except DCM, which was dried on basic alumina for use in resin preparation. Deuterated solvents, dimethyl sulphoxide ($d_6$-DMSO), methanol (MeOH) ($CD_3OD$), chloroform ($CDCl_3$) and dimethyl formamide ($d_7$-DMF) were purchased from Cambridge Isotope Laboratories.

### 2.3 Instrumentation

Solid phase peptide synthesis was performed using machine assisted protocols on a Quartet Symphony Peptide Synthesiser (Protein Technologies, Inc.). Each cycle of addition involved washing with DCM, followed by DMF, draining the reaction vessel between washes; deprotection with 20% piperidine/DMF for 3 min, draining the reaction vessel, then deprotection for 17 min, draining the reaction vessel; a DMF wash; a DCM wash; two DMF washes, draining the reaction vessel between washes; coupling for 3.5 h; draining the reaction vessel; a DMF wash, drain; a DCM wash, drain. In an average experiment Fmoc-py-COOH (0.36 g, 1 mmol) and aliphatic linkers (2 mmol) were mixed in a small beaker with HBTU (0.9 equiv.), DMF (2 mL), NMP (5 mL), DIEA (1 mL) and the resulting solution stirred for 5 min. The activated acid was then transferred manually via syringe into the reaction vessel. Quartet® software was used for operation of the peptide synthesiser. Coupling protocols and wash cycles were created using customised cleavage programs.

Catalytic hydrogenation reactions were conducted using a Parr Shaker Type Hydrogenation Apparatus (GE Motors and Industrial Systems). Reactions were carried out in a 500 mL reaction vessel with a steel enclosure. Before commencing the reaction, the hydrogen reservoir was filled to 50 psi of hydrogen and the reaction bottle evacuated 3 times to remove air. The pressure in the hydrogen reservoir was brought back to 50 psi and was then allowed to equilibrate with the reaction bottle. The solution was shaken for 5 h and the gas was then vented slowly.
Routine NMR spectra were performed on a 300 MHz Varian Mercury spectrometer equipped with a variable temperature pulsed field gradient probe and analysed using the Windows® based software MestReC (Version 4.5.6). In a typical experiment for one-dimensional $^1$H spectra, a spectral width of 4,500 Hz was used with 18,000 data points and relaxation delay of 1 s, with the temperature kept constant at 25 °C (CDCl$_3$) or 35 °C ($d_6$-DMSO), depending on the solvent used. Two dimensional NOESY spectra were accumulated using a 4,200 Hz spectral width with 256 increments in the t1 dimension, 2,048 points in the t2 dimension and mixing times between 300-800 ms. $^{195}$Pt NMR were obtained on a 400 MHz Bruker Avance spectrometer, either in $d_6$-DMSO, $d_7$-DMF or D$_2$O, referenced externally to K$_2$PtCl$_4$ (-1631 ppm). The number of transients used in any given experiment varied in each case with depending on the sample concentration and signal strength.

Positive ion ESI mass spectra were acquired using a Micromass (Wyntheshawe, UK) Quattro Micro™ spectrometer equipped with a Z-spray probe. Solutions of H$_2$O and acetonitrile (ACN) containing concentrations ranging between 10 and 50 μM were injected into the instruments at a flow rate of 10 μL min$^{-1}$. The source and desolvation temperatures were 150 and 120 °C respectively. The capillary tip potential and cone voltage were 2500 and 50 V respectively. Between 10 and 50 acquisitions were summed to obtain spectra, which were calibrated against a standard CsI solution (750 mM) over the same m/z range.
2.4 Solution Phase Synthesis Experimental

2.4.1 1-Methyl-4-nitropyrrrole-2-carboxylic acid

Ac₂O (20 mL) was treated with HNO₃ (4 mL, 70%) in an ice bath and the mixture heated to 50 °C for 15 min, then cooled to room temperature, before it was slowly added to a suspension of 1-methyl-2-pyrrole carboxylic acid (4.00 g, 32 mmol) in Ac₂O (12 mL, at -25 °C). The mixture was stirred at -15 °C for 0.5 h, then room temperature for a further 20 min. The mixture was again cooled to -25 °C and the resultant precipitate collected in a funnel cooled with dry ice. The solid was washed with a small quantity of cold Ac₂O (-25 °C). The crystalline solid was dissolved in 1 M sodium hydroxide. Acidification with 1 M hydrochloric acid precipitated the pure compound (2.90 g, 53%). ¹H NMR 300 MHz, (d₆-DMSO) δ 8.19 (d, 1H, J = 2.0 Hz, H1), 7.23 (d, 1H, J = 2.0 Hz, H2), 3.90 (s, 3H, CH₃).

2.4.2 Methyl 1-methyl-4-nitropyrrrole-2-carboxylate

A cold solution of sulphuric acid (H₂SO₄) (2.9 mL) in MeOH (29.0 mL) was added to 1-methyl-4-nitropyrrrole-2-carboxylic acid (2.90 g, 17 mmol). The mixture was heated at reflux for 24 h. Water (50 mL) was added and the mixture extracted with CDCl₃ (3 × 50 mL). The organic layer was dried over magnesium sulphate, and the solvent evaporated under reduced
pressure to afford a white solid (2.43 g, 78%). $^1$H NMR 300 MHz, $\left(d_6\right)$-DMSO $\delta$ 8.26 (d, 1H, $J = 2.1$ Hz, H1), 7.31 (d, 1H, $J = 2.0$ Hz, H2), 3.93 (s, 3H, CH$_3$), 3.80 (s, 3H, OCH$_3$).

### 2.4.3 Methyl 4-(1-methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxylate (PyPyCO$_2$CH$_3$)

![Methyl 4-(1-methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxylate (PyPyCO$_2$CH$_3$)](image)

Methyl-1-methyl-4-nitropyrrrole-2-carboxylate (0.50 g, 2.70 mmol) in MeOH (64 mL) and Pd/C (10%, 50 mg) was stirred under H$_2$ (1 atm) until the TLC (2% MeOH / DCM) indicated no starting material remained (1 h). The mixture was filtered through celite to remove the catalyst and DMF (3 mL) was added. MeOH was removed under reduced pressure and 1-methyl-2-pyrrole carboxylic acid (1 equiv., 0.34 g, 2.70 mmol), HOBt (1.5 equiv. 0.55 g, 4.05 mmol), TBTU (1.5 equiv., 1.30 g, 4.05 mmol), and TEA (5 equiv. 1.37 g, 13.50 mmol) was added and the mixture stirred under nitrogen for 16 h. The residue was purified by flash chromatography (2% MeOH/DCM) yielding a light brown solid (0.360 g, 48.0%). $^1$H NMR 300 MHz, (CD$_3$OD) $\delta$ 7.97 (s, 2H), 7.84 (d, 1H, $J = 2.0$ Hz), 7.34 (d, 1H, $J = 2.0$ Hz), 6.93 (s, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.79 (s, 3H).

### 2.4.4 4-(1-Methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxylic acid (PyPyCO$_2$H)

![4-(1-Methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxylic acid (PyPyCO$_2$H)](image)
To PyPyCO₂CH₃ (360 mg, 1.38 mmol) in THF/MeOH (1.1/7.5 mL) was added lithium hydroxide (1 M, 5.5 mL) and the solution stirred at 60 °C (oil bath) for 1.5 h and monitored by TLC (10% MeOH/DCM). The solvent was removed under reduced pressure, the solution cooled and acidified with hydrochloric acid (1 M, 5.0 mL). The solid was collected and air dried and left in a desiccator under vacuum overnight (250 mg, 69.3%). ¹H NMR 300 MHz, (d₆-DMSO) δ 9.75 (s, 1H, H5), 7.97 (s, 1H, H3), 7.52 (s, 1H, H4), 7.40 (s, 1H, H2), 6.92 (s, 1H, H6), 6.83 (s, 1H, H7), 3.91 (s, 3H, H8), 3.89 (s, 3H, H1).

2.4.5 Methyl 4-[4-(1-methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxyamido]-N-methylpyrrole-2-carboxylate

(PyPyPyCO₂CH₃)

Methyl-1-methyl-4-nitropyrrrole-2-carboxylate (160 mg, 0.87 mmol) in MeOH (30 mL) and Pd/C (10%, 16 mg) were stirred under H₂ (1 atm) until TLC analysis (2% MeOH / DCM) indicated that no starting material remained (1 h). The mixture was filtered through celite to remove the catalyst and DMF was added (3 mL). MeOH was removed under reduced pressure and PyPyCO₂H (1 equiv., 215 mg, 0.87 mmol) HOBt (1.5 equiv., 177 mg, 1.31 mmol) TBTU (1.5 equiv. 421 mg, 1.31 mmol) and Et₃N (5 equiv. 440 mg, 4.35 mmol) was added and the solution left to stir for 16 h. The residue was purified by flash chromatography (2% MeOH/DCM) yielding a light brown solid (44 mg, 13.2%). ¹H NMR 300 MHz, (CD₃OD) δ 10.03 (s, 1H), 8.09 (s, 1H), 7.35 (s, 1H), 7.20 (s, 1H), 6.90 (s, 1H), 6.85 (s, 1H), 6.10 (s, 1H), 5.50 (s, 1H), 3.90 (s, 6H), 3.80 (s, 3H), 3.40 (s, 3H).
2.4.6 4-[4-(1-Methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxyamido]-N-methylpyrrole-2-carboxylic acid
(\text{PyPyPyCO}_2\text{H})

To PyPyPyCO\textsubscript{2}CH\textsubscript{3} (40.0 mg, 0.10 mmol) in THF/MeOH (1.1/7.5 mL) was added lithium hydroxide (1 M, 5.5 mL) and the solution stirred at 60 °C for 1.5 h and monitored by TLC analysis (10% MeOH/DCM). The solvent was removed under reduced pressure, the solution cooled and acidified with hydrochloric acid (1 M, 5 mL). The solid was collected and air dried and left in a desiccator under vacuum overnight (18.9 mg, 49.0%). \textsuperscript{1}H NMR 300 MHz, (\textit{d}_6-\text{DMSO}) \text{δ} 9.85 (s, 1H, H5), 9.82 (s, 1H, H9), 7.40 (s, 1H, H11), 7.20 (s, 1H, H7), 7.00 (s, 1H, H6), 6.90 (s, 1H, H2), 6.83 (s, 1H, H4), 6.80 (s, 1H, H10), 6.00 (s, 1H, H3), 3.83 (s, 3H, H1), 3.82 (s, 3H, H8), 3.81 (s, 3H, H12).

2.5 Solution Phase Synthesis Discussion

The general procedure for peptide synthesis involves stepwise elongation and segment condensation. This principle has been adopted to synthesise polyamides in the past and has also been applied to solid phase supports.\textsuperscript{155} The synthesis of the natural polyamides Nt and Dst by Lown illustrated these basic principles.\textsuperscript{166} In the present study, the synthesis of the polyamides in this project began with solution based reactions with the coupling agents HO\textit{B}t and TBTU (Figure 2.3). HO\textit{B}t and TBTU work by activation of the carboxylic acid group; formation of a more reactive intermediate that is susceptible to nucleophilic attack. The acid is first deprotonated with base producing the nucleophilic carboxylate which attacks
TBTU and after several rearrangements the active ester is formed and reacted with the free amine (Figure 2.3).

![Activation of carboxyl groups for peptide and coupling reaction using HOBt and TBTU. Inset: The coupling agents a HOBt and b TBTU.](image)

**Figure 2.3** Activation of carboxyl groups for peptide and coupling reaction using HOBt and TBTU. Inset: The coupling agents a HOBt and b TBTU.

The total synthesis of the three ring polyamide 4-[4-(1-methylpyrrole-2-carboxyamido)-N-methylpyrrole-2-carboxyamido]-N-methylpyrrole-2-carboxylic acid (PyPyPyCO₂H) is shown in Scheme 2.1. The first step in solution phase synthesis was nitration of 1-methyl-2-pyrrole carboxylic acid with Ac₂O and HNO₃. HNO₃ was first added to acetic anhydride dropwise in an ice bath, as the formation of the nitronium ion is dangerously exothermic if left at room temperature. This mixture was then added dropwise to 1-methyl-2-pyrrole carboxylic acid at -25 °C and then allowed to stir for 30 min, and another 20 min at room temperature. The solution was again cooled to -25 °C and the resulting precipitate collected in a funnel cooled.
in dry ice. Several variations of the cooled funnel were tried; the most successful method simply used a fluted filter paper that was placed inside a funnel filled with dry ice.

The collected product was then washed with Ac₂O, taken up in sodium hydroxide and precipitated with hydrochloric acid. Comparison of the \(^1\text{H} \text{NMR}\) of the product (1-methyl-4-
nitopyrrole-2-carboxylic acid) and that of the starting material shows the disappearance of the one aromatic singlet leaving only two aromatic singlet resonances present, which is consistent with nitration at the 4-position (Figure 2.4).

Figure 2.4 $^1$H NMR spectrum of 1-methyl-4-nitropyrrrole-2-carboxylic acid, 300 MHz, 35 °C, $d_6$-DMSO.

The NOESY spectrum of 1-methyl-4-nitropyrrrole-2-carboxylic acid is shown in Figure 2.5. An NOE resonance between the methyl group at 3.90 ppm and the H(1) proton at 8.19 ppm can be seen. Long range coupling of H(1) and the H(2) proton at 7.23 ppm is also present despite the presence of a nitro group between them. It is hypothesised this long range NOE might be a result of stacking of the pyrrole rings in an anti-parallel manner which could lead to an intermolecular NOE.

The next step was to protect the carboxyl group by forming the methyl ester which prevents polymerisation during coupling reactions. The ester was made by heating at reflux 1-methyl-4-nitropyrrrole-2-carboxylic acid in $\text{H}_2\text{SO}_4$ and MeOH for 24 h, followed by extraction with CHCl$_3$. 
Figure 2.5 NOESY of 1-methyl-4-nitropyrrrole-2-carboxylic acid, 300 MHz, 35 °C, $d_6$-DMSO.

Analysis of the $^1$H NMR shows the appearance of the extra methyl singlet (3.80 ppm) after esterification and the presence of some impurities which appear to be solvent (Figure 2.6). The NOESY spectrum for methyl 1-methyl-4-nitropyrrrole-2-carboxylate is shown in Figure 2.7. A resonance between the H(1) proton at 8.26 ppm and the methyl group at 3.93 ppm can be seen. The other methyl group present at 3.8 ppm (integrates to 3H) can therefore be assigned as the methyl ester (OCH$_3$). Coupling between the H(1) and H(2) protons is seen again as in the NOESY for 1-methyl-4-nitropyrrrole-2-carboxylic acid. Stacking effects are once again assumed for this anomaly.
Figure 2.6 \(^1\text{H NMR}\) spectrum of methyl 1-methyl-4-nitropyrole-2-carboxylate, 300 MHz, 35 °C, \(d_6\)-DMSO. The solvent impurities are denoted by the arrows.

Figure 2.7 NOESY of methyl 1-methyl-4-nitropyrole-2-carboxylate, 300 MHz, 35 °C, \(d_6\)-DMSO.
The first coupling reaction was achieved with 1-methyl-2-pyrrole carboxylic acid and methyl-1-methyl-4-nitropyrrrole-2-carboxylate. The amine was made via hydrogenation of the nitro-pyrrole immediately prior to the coupling reaction, as the stability of the free amine was low. Hydrogenation with Pd/C (1 atm), for approximately 1 h (monitored by TLC analysis), yielded the amine. The catalyst was removed with celite, DMF was added, and the solvent (MeOH) evaporated under reduced pressure. It was necessary to reduce exposure to the atmosphere as pyrrole amines are unstable and revert back to the nitro species rapidly upon contact with air. 1-Methyl-2-pyrrole carboxylic acid was then coupled to methyl 1-methyl-4-aminopyrrrole-2-carboxylate using HOBT and TBTU over 16 h. The residue was then loaded onto a silica column, eluted using 2% MeOH/DCM, and the eluent collected in fractions. Despite optimisations of the solvent system the product could not be obtained with high purity as seen with the $^1$H NMR of PyPyCO$_2$CH$_3$ (Figure 2.8).

![Figure 2.8](image)

**Figure 2.8** $^1$H NMR spectrum of PyPyCO$_2$CH$_3$, 300 MHz, 35 °C, CD$_3$OD. The impurities are denoted by the arrows.

Removal of the methyl ester and formation of the carboxylic acid via precipitation with HCl greatly improved the purity of the sample; the NMR spectra showed only minor impurities
were present and the majority of the contaminants from the previous step had disappeared (Figure 2.9).

**Figure 2.9** ¹H NMR spectrum of PyPyCO₂H, 300 MHz, 35 °C, d₆-DMSO. The impurities are denoted by the arrows.

The NOESY spectrum for PyPyCO₂H is shown in Figure 2.10. The experiment shows the methyl group at 3.89 ppm (H1) is coupled to the proton at 7.40 ppm which is assigned as H(2). H(2) shows an NOE with the H(3) proton at 7.97 ppm. H(3) is coupled to the proton at 7.52 ppm designated H(4). Long range coupling is seen between the H(4) proton and the characteristically downfield amide proton (9-11 ppm) at 9.75 ppm assigned as H(5). The H(5) proton couples to the two remaining pyrrole protons on the last ring; the proton at 6.92 ppm, H(6), and the resonance at 6.83 ppm, H(7). H(6) shows coupling to the remaining N-methyl group at 3.91 ppm, H(8). Two impurities are seen in the spectrum which are assumed
to be unreacted starting material which were unable to be removed by column chromatography or during de-esterification.

Figure 2.10 $^{1}$H NMR spectrum of PyPyCO$_2$H, 300 MHz, 35 °C, $d_6$-DMSO.

The coupling of the third pyrrole ring to the polyamide using the same coupling agents (HOBt and TBTU) yielded an impure product as with the previous coupling reaction. Purification via column chromatography also failed to remove the impurities. The $^{1}$H NMR spectrum of PyPyPyCO$_2$CH$_3$ is shown in Figure 2.11. The NMR spectrum shows that most of the impurities are aromatic in origin, and is hypothesised to be unreacted coupling agent that was not removed during column purification. Both HOBt and TBTU have 4 aromatic protons each and two aromatic amine protons each and by the appearance of the cluttered NMR spectrum is the most likely source of impurities present. The contamination of the product with unreacted starting materials methyl 1-methyl-4-nitropyrrrole-2-carboxylate and PyPyCOOH is also likely although the number of methyl groups at approximately 3.6-4.0 ppm suggests the abundance of N-methyl pyrrole based impurities is low.
Figure 2.11 $^1$H NMR spectrum of PyPyPyCO$_2$CH$_3$, 300 MHz, 35 °C, $d_6$-DMSO. The impurities are denoted by the arrows.

Ester hydrolysis of PyPyPyCO$_2$CH$_3$ and precipitation with HCl produced PyPyPyCO$_2$H, which again saw, by $^1$H NMR, that most of the contaminants that were water soluble removed (Figure 2.12). The NOESY spectrum of PyPyPyCO$_2$H is shown in Figure 2.13. The methyl group at 3.83 ppm is assigned as H(1), and shows coupling to the aromatic proton at 6.90 ppm, H(2). The H(2) proton shows coupling to the proton at 6.00 ppm designated as H(3). H(3) shows coupling to the proton at 6.83 ppm and is assigned as H(4). H(4) couples to the amide proton at 9.85 ppm and is designated H(5). Coupling from H(5) to the aromatic proton at 7.00 ppm, which is assigned as H(6). The H(6) proton shows coupling to the remaining amide proton at 9.82 ppm assigned as H(9). H(9) shows coupling to the aromatic proton at 6.80 ppm, assigned H(10). The remaining two coupling systems; the protons at 7.20 ppm, 3.82 ppm, H(7)/H(8), and the protons at 7.40 ppm, 3.81 ppm, H(11)/H(12) show no couplings to other expected proton resonances. However, it is hypothesised that the molecules are forming dimers which is a common occurrence with these compounds. The
dimerised NOEs seen include, H(11)/H(5), H(7)/H(9), H(2)/H(9) and H(3)/H(12). These NOEs give evidence to an antiparallel dimerisation model where the NOEs are occurring between protons that are equidistant from the center of the molecule.

Figure 2.12 $^1$H NMR spectrum of PyPyPyCO$_2$H, 300 MHz, 35 °C, $d_6$-DMSO. The impurity is denoted by the arrow.

Figure 2.13 NOESY spectrum of PyPyPyCO$_2$H, 300 MHz, 35 °C, $d_6$-DMSO.
The $^1$H NMR data for the solution based experiments are summarised in Table 2.1.

**Table 2.1 $^1$H NMR Data for Solution Phase Chemistry**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proton Assignment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Amide</td>
</tr>
<tr>
<td>1-Methyl-4-nitropyrrle-2-carboxylic acid</td>
<td>8.19 (d, 1H, $J = 2.0$ Hz, H1) 7.23 (d, 1H, $J = 2.0$ Hz, H2)</td>
</tr>
<tr>
<td>Methyl 1-methyl-4-nitropyrrle-2-carboxylate</td>
<td>8.26 (d, 1H, $J = 2.1$ Hz, H1) 7.31 (d, 1H, $J = 2.0$ Hz, H2)</td>
</tr>
<tr>
<td>PyPyCO$_2$CH$_3$</td>
<td>7.97 (s, 2H) 7.84 (d, 1H, $J = 2.0$ Hz) 7.34 (d, 1H, $J = 2.0$ Hz) 6.93 (s, 1H)</td>
</tr>
<tr>
<td>PyPyCO$_2$H</td>
<td>9.75 (s, 1H, H5) 7.97 (s, 1H, H3) 7.52 (s, 1H, H4) 7.40 (s, 1H, H2) 6.92 (s, 1H, H6) 6.83 (s, 1H, H7)</td>
</tr>
<tr>
<td>PyPyPyCO$_2$CH$_3$</td>
<td>10.03 (s, 1H) 8.09 (s, 1H) 6.85 (s, 1H) 7.35 (s, 1H) 6.10 (s, 1H) 7.20 (s, 1H) 6.90 (s, 1H) 5.50 (s, 1H)</td>
</tr>
<tr>
<td>PyPyPyCO$_2$H</td>
<td>9.85 (s, 1H, H5) 9.82 (s, 1H, H9) 7.40 (s, 1H, H11) 7.20 (s, 1H, H7) 7.00 (s, 1H, H6) 6.90 (s, 1H, H2) 6.83 (s, 1H, H4) 6.80 (s, 1H, H10) 6.00 (s, 1H, H3)</td>
</tr>
</tbody>
</table>

Further coupling of additional pyrrole rings to the three ring polyamide PyPyPyCO$_2$H was unsuccessful. Column chromatography of the reaction mixture and $^1$H NMR analysis of the fractions did not show any evidence for the addition of a fourth pyrrole ring. Limited amounts of starting material available also meant reactions had to be done on small scales, making optimisation of the conditions difficult and time consuming. The coupling reactions that were carried out in solution phase gave many impurities in the crude product and purification via column chromatography was not effective. Although precipitation of the product after ester hydrolysis yielded compound that appeared to be of higher purity by NMR, reaction yields were significantly low which became the major concern as there was very little compound available to progress further. The difficulty in assigning protons for the
compound PyPyPyCO₂H was also encountered with dimerisation of the compound evident during the NOESY experiments. To extend beyond three ringed polyamides a new synthetic approach was thus necessary and solid phase was introduced as an alternative method in this project.
2.6 Pyrrole Monomer Synthesis for Solid Phase

2.6.1 Trichloroacetyl chloride

Trichloroacetyl chloride was made using an adaptation of the method of von Bosshard et al.\textsuperscript{167} Trichloroacetic acid (50.0 g, 0.3 mol), thionyl chloride (SOCl\textsubscript{2}, 25 mL, 0.33 mol) and DMF (2 mL) were combined, purged with N\textsubscript{2}(g), and heated for 2 h at 80 °C. The solution was then distilled (114-117 °C) yielding a colourless liquid (42.1 g, 85.9%). IR sodium chloride (NaCl) 1750 cm\textsuperscript{-1} (C=O).

2.6.2 2-Trichloroacetyl-1-methylpyrrole (Py1)

2-Trichloroacetyl-1-methylpyrrole was synthesised using the method described by Nishiwaki et al.\textsuperscript{168} N-Methylpyrrole (10.0 g, 0.12 mol) in DCM (40 mL) was added dropwise to trichloroacetyl chloride (22.0 g, 0.12 mol) in DCM (40 mL) over 3 h and the solution left to stir overnight. The crude product was then reduced in volume (~40 mL) and purified using column chromatography (silica gel, 3:7 DCM/hexane) yielding the pale yellow solid (21 g, 75.5%). \textsuperscript{1}H NMR (300 MHz, CDCl\textsubscript{3}) δ 7.50 (dd, 1H, J = 1.6, 4.4 Hz, H2), 6.97 (m, 1H, H4), 6.22 (dd, 1H, J = 2.4, 4.4 Hz, H3), 3.97 (s, 3H, H1).
2.6.3 4-Nitro-2-trichloroacetyl-1-methylpyrrole (Py2)

![Chemical structure of 4-Nitro-2-trichloroacetyl-1-methylpyrrole (Py2)]

The synthesis of 4-nitro-2-trichloroacetyl-1-methylpyrrole was adapted from the method described by Hotzel et al.\textsuperscript{169} A mixture of HNO\textsubscript{3} (70%, 24 mL) and Ac\textsubscript{2}O (75 mL) was added dropwise over 30 min to a solution of 2-trichloroacetyl-1-methylpyrrole (30 g, 0.13 mol) in Ac\textsubscript{2}O (75 mL cooled to -40 °C). The solution was warmed to room temperature and left to stir for 2 h. The mixture was then cooled to -20 °C, iso-propanol (125 mL) added, and then left to stir at -20 °C overnight. The solid was then collected under vacuum, and washed with cold iso-propanol and dried under vaccum. (20.8 g, 57.9%). \textsuperscript{1}H NMR 300 MHz, \((d_6\text{-DMSO})\) \(\delta\) 8.55 (d, 1H, \(J = 1.2\) Hz, H2), 7.81 (d, 1H, \(J = 1.8\) Hz, H3), 4.00 (s, 3H, H1).

2.6.4 tert-Butyl 4-nitro-1-methylpyrrole-2-carboxylate (Py3)

![Chemical structure of tert-Butyl 4-nitro-1-methylpyrrole-2-carboxylate (Py3)]

tert-Butyl 4-nitro-1-methylpyrrole-2-carboxylate was synthesised using an adaptation of the method by Wurtz et al.\textsuperscript{155} Sodium tert-butoxide (10.40 g, 0.11 mol) was added over 1 h to a suspension of 4-nitro-2-trichloroacetyl-1-methylpyrrole (20.81g, 0.08 mol) in 210 mL of tert-butyl alcohol. The suspension was then heated at reflux under N\textsubscript{2}(g) for 5 h and the reaction
quenched by addition of water (500 mL) and the product extracted with CDCl$_3$, dried with MgSO$_4$, and reduced under vacuum. (13.8 g, 75%). $^1$H NMR 300 MHz, (d$_6$-DMSO) $\delta$ 8.20 (d, 1H, $J = 2.3$ Hz, H2), 7.23 (d, 1H, $J = 2.3$ Hz, H3), 3.90 (s, 3H, H1), 1.53 (s, 9H, H4).

2.6.5 tert-Butyl 4-[(9-fluorenylethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate (Py4)

$t$ert-Butyl-4-Fmoc-1-methylpyrrole-2-carboxylate was synthesised using methods adapted from Wurtz et al.$^{155}$ To a solution of tert-butyl-4-nitro-1-methylpyrrole-2-carboxylate (20 g, 0.09 mol) in 100 mL of DMF was added 10% Pd/C (2 g). The mixture was stirred for 4 h under hydrogen (50 psi) and filtered through celite, washed with DMF (200 mL) and the filtrates combined. Fmoc-Cl (25.3 g, 0.10 mol) and DIEA (35 mL, 0.20 mol) were added to the solution and stirred overnight. Water (250 mL) was added, and the mixture extracted with diethyl ether (2 $\times$ 500 mL). The ether was dried (magnesium sulphate) and concentrated in vacuo to $\sim$200 mL. Hexane was added causing the precipitate of a brown solid which was collected, washed with cold MeOH/H$_2$O yielding a white solid. (19.51 g, 52.80%). $^1$H NMR 300 MHz, (d$_6$-DMSO) $\delta$ 9.40 (s, 1H, H7), 7.90 (d, 2H, $J = 7.3$ Hz, H1), 7.71 (d, 2H, $J = 7.4$ Hz, H4), 7.42 (t, 2H, $J = 7.4$ Hz, H2), 7.34 (t, 2H, $J = 7.4$ Hz, H3), 7.00 (s, 1H, H9), 6.61 (s,1H, H8), 4.43 (d, 2H, $J = 5.8$ Hz, H6), 4.28 (d, 1H, $J = 6.0$ Hz, H5), 3.76 (s, 3H, H10), 1.48 (s, 9H, H11).
2.6.6 4-[(9-Fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid (Py5)

4-Fmoc-1-methylpyrrole-2-carboxylic acid was synthesised using methods adapted from Wurtz et al.\textsuperscript{155} tert-Butyl-4-Fmoc-1-methylpyrrole-2-carboxylate (5.0 g, 12 mmol) was dissolved in 100 mL of DCM and the solution cooled to 0 °C. [TiCl\textsubscript{4}] (25 mL, 1.0 M in DCM) was added dropwise under \textsubscript{N}2(\textsubscript{g}) and the mixture stirred for 30 min. HCl (1 M, 250 mL) was cooled to 4 °C and added dropwise. The resulting white precipitate was filtered, washed with cold water and air dried. (3.47 g, 79.8%). \textsuperscript{1}H NMR 300 MHz, (\textit{d}\textsubscript{6}-DMSO) δ 9.37 (s, 1H, H7), 7.89 (d, 2H, \textit{J} = 7.3 Hz, H1), 7.70 (d, 2H, \textit{J} = 7.4 Hz, H4), 7.43 (t, 2H, \textit{J} = 7.4 Hz, H2), 7.35 (t, 2H, \textit{J} = 7.4 Hz, H3), 7.03 (s, 1H, H9), 6.63 (s, 1H, H8), 4.44 (d, 2H, \textit{J} = 5.8 Hz, H6), 4.27 (t, 1H, \textit{J} = 6.0 Hz, H5), 3.78 (s, 3H, H10).
2.7 Discussion of Pyrrole Monomer Synthesis

Production of the pyrrole monomer precursors required for solid phase synthesis of LLSP4-Pt were made using adaptations of various literature methods.\(^{155, 167-169}\) Py1 was afforded by trichloroacetylation of 1-methylpyrrole and subsequent nitration at the 4 position provided Py2. Conversion to the tert-butyl ester yielded Py3 which was reduced, and the addition of Fmoc-Cl and base, produced Py4. The tert-butyl group was removed with TiCl\(_4\) and the product Py5 was precipitated by the addition of HCl. The total synthesis of the pyrrole monomer is described in Scheme 2.2.

Synthesis of the Fmoc-py-acid monomer began with the preparation of trichloroacetyl chloride. Trichloroacetyl chloride was prepared immediately before usage via acyl halide formation of the carboxylic acid using thionyl chloride, with DMF as the catalyst. It was necessary to ensure the solvents were dried prior to use; trichloroacetic acid was dried by repeated azeotropic evaporations from benzene under reduced pressure and left under vacuum for several hours; thionyl chloride was dried via distillation from linseed oil and stored in a vacuum desiccator.

![Scheme 2.2](image-url)  

**Scheme 2.2** Total synthesis of the pyrrole monomer 4-[(9-fluorenylmethoxycarbonyl)-amino]-1-methylpyrrole-2-carboxylic acid (Py5).
The reaction between trichloroacetic acid and thionyl chloride was allowed to proceed for 2 h and followed by distillation of the clear product where the first ~10 mL was discarded and the product collected until ~10 mL remained undistilled to ensure purity. Infra-red analysis of distilled product shows a carbonyl stretch at 1750 cm$^{-1}$ (the carboxylic acid impurity appears at 1820 cm$^{-1}$).

Trichloroacylation of 1-methylpyrrole was achieved via dropwise addition of trichloroacetyl chloride in DCM, which was dried with basic alumina before use. The resulting purple reaction mixture was allowed to stir overnight and then purified on a silica column. Loading of the column was done slowly with the column unpressurised to avoid compaction of the reaction mixture; this is known to cause problems with solvent flow through the column as a result. Elution with hexane/DCM caused the product to elute first as a pale yellow band, while the purple band migrated at approximately half the rate of the product. $^1$H NMR of Py1 in CDCl$_3$ (Figure 2.14) shows the expected aromatic proton resonances; two double doublets at 6.22 and 7.50 ppm and a multiplet at 6.97 each signal integrating to 1 proton. The NOESY spectrum for Py1 is shown in Figure 2.15. An NOE crosspeak is seen between the methyl group at 3.97 ppm, H(1), and an aromatic proton at 7.50 ppm, H(2). The H(2) proton also shows coupling to the resonance at 6.22 ppm and is assigned as H(3). The remaining proton at 6.97 ppm is assigned as H(4).

Nitration of Py1 at the 4 position was achieved by addition of a mixture of HNO$_3$ and Ac$_2$O. The Ac$_2$O was used fresh from sealed bottles to ensure dryness as the reaction equilibrium relies heavily upon a moisture free environment. Preparation of the nitronium ion (NO$_2^+$) is achieved by drawing water from the solution (formation of acetic acid from Ac$_2$O) and driving the equilibrium to its formation. It was crucial that the HNO$_3$/Ac$_2$O mixture was prepared by dropwise addition of the acid to a solution of Ac$_2$O cooled to –10 °C to ensure there was no rapid formation of NO$_2^+$.
Figure 2.14 $^1$H NMR spectrum of Py1, 300 MHz, 25 °C, CDCl$_3$.

Figure 2.15 NOESY of Py1, 300 MHz, 25 °C, CDCl$_3$. 
Addition at room temperature results in a vigorous reaction that expends NO$_2^+$ as it is an unstable ion. Once all the HNO$_3$ has been added to the Ac$_2$O it is allowed to slowly rise to room temperature and the mixture added dropwise to the solution of trichloroacetyl-1-methylpyrrole cooled to -40 °C. Problems with condensation of water inside the flask at low temperatures can be avoided by purging the flask with N$_2$(g) as the addition is carried out.

Characterisation of Py2 by $^1$H NMR in $d_6$-DMSO shows the disappearance of an aromatic proton seen in the spectra of Py1 indicating nitration has occurred (Figure 2.16). The NOESY spectra for Py2 is shown in Figure 2.17. An NOE crosspeak is seen between the methyl group at 4.00 ppm and an aromatic proton at 8.55 ppm, assigned as H(1) and H(2) respectively. The remaining proton at 7.81 ppm is designated as H(3).

![Figure 2.16](image)

**Figure 2.16** $^1$H NMR spectrum of Py2, 300 MHz, 35 °C, $d_6$-DMSO.

Esterification of Py2 was carried out in tert-butanol which was stored and kept dry in a vacuum desiccator. It was important to also keep the solid sodium tert-butoxide dry as the reaction is highly moisture sensitive; the powder was dried overnight, under vacuum at 50
°C, in a drying oven. When sodium tert-butoxide added slowly over 1 h to the reaction vessel it was added via a powder addition funnel under N\textsubscript{2}(g). The \textsuperscript{1}H NMR spectrum of Py3 in \textit{d}\textsubscript{6}-DMSO shows the appearance of tert-butyl methyl groups resonating at 1.48 ppm, H(4), and integrating to 9 protons as expected (Figure 2.18).

![Figure 2.17](image)

**Figure 2.17** NOESY of Py2, 300 MHz, 35 °C, \textit{d}\textsubscript{6}-DMSO.

The NOESY spectrum for Py3 is shown in Figure 2.19. The methyl proton at 3.90 ppm, H(1) shares an NOE crosspeak with the resonance at 8.20 ppm, designated H(2). The remaining aromatic proton at 7.23 ppm is assigned as H(3).

Hydrogenation of Py3 was achieved under 50 psi of hydrogen for 4 h although it was also sufficient to hydrogenate overnight (~15 h) at 30 psi. Washing the celite with DMF prior to filtration of the catalyst was necessary and prevented Pd/C from contaminating the sample. During the filtration, caution was taken not to let the celite dry out as Pd/C could ignite. Filtration of the product was done quickly and Fmoc-Cl and DIEA added without delay as the free amine of Py3 is unstable and reverts to the nitro species when exposed to air.
Figure 2.18  $^1$H NMR spectrum of Py3, 300 MHz, 35 °C, $D_6$-DMSO.

Figure 2.19  NOESY of Py3, 300 MHz, 35 °C, $D_6$-DMSO.
The $^1$H NMR spectrum of Py4 in $d_6$-DMSO is shown in Figure 2.20 while the NOESY spectrum is shown in Figure 2.21. The resonance at 7.90 ppm, designated H(1), couples to the proton at 7.42 ppm, H(2). H(2) shares a crosspeak with the resonance at 7.34 ppm and is assigned as H(3), while H(3) is in turn coupled to the proton at 7.71 ppm, H(4). The H(4) proton neighbours the aliphatic resonance at 4.28 ppm, which is designated H(5). The resonance at 4.43 is assigned as H(6) as it shares NOE crosspeaks with H(5) and the easily identifiable amide proton at 9.40 ppm, H(7). The H(5) amide protons shows NOE crosspeaks with two resonances; 7.00 ppm, H(9) and 6.61 ppm, H(8). H(9) couples to the methyl group at 3.76 and is assigned as H(10). The remaining aliphatic proton at 1.48 ppm, which integrates to 9 protons, is assigned as H(11).

![Figure 2.20 $^1$H NMR spectrum of Py4, 300 MHz, 35 °C, $d_6$-DMSO.](image)

The complete $^1$H NMR assignment of Py5 in $d_6$-DMSO is given in Figure 2.22 with the NOESY spectrum detailed in Figure 2.23. The spectral assignment was identical to that of Py4, although the protons showed minor chemical shifts between analogous resonances. The
resonances at 7.89, 7.43, 7.35 and 7.70 ppm each integrate to 2H, indicative of the Fmoc protons were assigned as H(1), H(2), H(3) and H(4) respectively.

Figure 2.21  NOESY spectrum of Py4, 300 MHz, 35 °C, D$_6$-DMSO.

H(4) shares an NOE with the 4.27 ppm resonance assigned H(5), which couples to the H(6) proton present at 4.43 ppm. The far downfield singlet at 9.37 ppm is assigned as the amide proton H(7) which also shows coupling to H(6). Additionally, H(7) shares NOEs with both singlets at 6.63 and 7.03 ppm which are assigned as the pyrrole aromatic protons. H(9) is assigned as the singlet at 7.03 as it shares an NOE with the H(10) singlet at 3.78 ppm (3H). The remaining singlet at 6.63 ppm is therefore assigned as H(8). The NMR data is summerised in Table 2.2.
Figure 2.22  $^1$H NMR spectrum of Py5, 300 MHz, 35 °C, $d_6$-DMSO.

Figure 2.23  NOESY of Py5, 300 MHz, 35 °C, $d_6$-DMSO.
The Fmoc protected pyrrole monomer, Py5, was successfully synthesised in high yields for use in solid phase chemistry. A requirement for peptide synthesis, using solid phase supports, is a monomeric building block with a protected amino group and a free carboxylic acid. In this project, protection of the amine was afforded by an Fmoc group which prevents polymerisation during coupling. Fmoc remains stable in acidic conditions but is removed under basic conditions. The free acid on Py5 enables the monomer to be coupled to an amine on the resin. Once the monomer has been coupled, the Fmoc protecting group can removed with base and the next monomer can be added providing a simple and sequential method of peptide chain development.

Table 2.2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proton Assignment</th>
<th>Aliphatic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amide</td>
<td>Aromatic</td>
</tr>
<tr>
<td>Py1</td>
<td>7.50 (dd, 1H, (J = 1.6) Hz, (J = 4.4) Hz, H2)</td>
<td>6.97 (m, 1H, H4)</td>
</tr>
<tr>
<td>Py2</td>
<td>8.55 (d, 1H, (J = 1.2) Hz, H2)</td>
<td>7.81 (d, 1H, (J = 1.8) Hz, H3)</td>
</tr>
<tr>
<td>Py3</td>
<td>8.20 (d, 1H, (J = 2.3) Hz, H2)</td>
<td>7.23 (d, 1H, 2.3 Hz, H3)</td>
</tr>
<tr>
<td>Py4</td>
<td>7.90 (d, 2H, (J = 7.3) Hz, H1)</td>
<td>7.71 (d, 2H, (J = 7.4) Hz, H4)</td>
</tr>
<tr>
<td>Py5</td>
<td>7.89 (d, 2H, (J = 7.3) Hz, H1)</td>
<td>7.70 (d, 2H, (J = 7.4) Hz, H4)</td>
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</tbody>
</table>
2.8 Solid Phase Experimental

2.8.1 Synthesis of LLSP4-DPA Using Fmoc-β-Ala-OH-WANG resin

The polyamide LLSP4-DPA was prepared with machine assisted protocols using Fmoc-β-Ala-OH-WANG resin on a Quartet Symphony Peptide Synthesiser. WANG Resin was used as received. The resin was stored at 4 °C and dried under vacuum before use.

2.8.1.1 LLSP4-DPA Procedure

Fmoc-β-Ala-OH-WANG resin (0.8 g, 0.56 mmol) was weighed into a reaction vessel, washed and deprotected using Cleavage Protocol (I) (Table 2.3). During step 4 of (I), Pyrrole Activation Protocol (II) (Table 2.4) was initiated. After mixing for 5 min, the activated pyrrole solution was added to the reaction vessel and Coupling Protocol (III) (Table 2.5) was commenced. Protocols I to III were then repeated sequentially, two more times. Addition of the fourth pyrrole ring was achieved using protocols (I), Terminal Pyrrole Activation Protocol (IV) (Table 2.6) and then (III). The resin was washed with DCM (6 units, 30 s) and dried for 5 min and transferred to a 10 mL round bottom flask. 3-Dimethylaminopropylamine (3.2 mL, 25.3 mmol) was added to the resin and the solution heated at 55 °C for 18 h with gentle stirring. The resin was removed by filtration, and the product precipitated by addition of water (10 mL). The pale yellow product was filtered, washed with water and redissolved in ACN (10 mL), transferred to a round bottom flask and the solvent removed until ~1 mL remained. Water was added and the product was then lyophilised to yield a pale yellow solid. (87 mg, 24.03%) $^1$H NMR 300 MHz, ($d_6$-DMSO) δ 9.90 (s, 1H), 9.85 (s, 1H), 9.81 (s, 1H), 7.40 (t, 1H, $J = 5.4$ Hz), 7.85 (t, 1H, $J = 5.7$ Hz), 7.23 (d, 2H, $J = 1.8$ Hz), 7.19 (d, 1H, $J =$
1.8 Hz), 7.05 (t, 2H, J = 1.8 Hz), 6.94 (s, 1H), 6.93 (s, 1H), 6.83 (d, 1H, J = 1.8 Hz), 6.06 (t, 1H, J = 3.3 Hz), 3.87 (s, 3H), 3.86 (s, 3H), 3.85 (s, 3H), 3.80 (s, 3H), 3.36 (t, 2H, J = 6.9 Hz), 3.05 (t, 2H, J = 6.9 Hz), 2.33 (t, 2H, J = 7.2 Hz), 2.17 (t, 2H, J = 6.9 Hz), 2.12 (s, 3H), 2.09 (s, 3H), 1.51 (m, 2H, J = 7.2 / 14.4 Hz); ESI-MS: m/z: calcd for C32H42N10O5: 646.3; found: 647.0 [M+H]+.

Table 2.3  Cleavage Protocol (I)

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Units † † †</th>
<th>Mix time</th>
<th>Drain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>3</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>3 min</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>17 min</td>
<td>On</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>7</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
</tbody>
</table>

Table 2.4  Pyrrole Activation Protocol (II)

<table>
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<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Py-COOH</td>
<td>0.543 g, 1.49 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.540 g, 1.42 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.75 mL, 4.31 mmol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Mix 5 min</td>
</tr>
</tbody>
</table>

Table 2.5  Coupling Protocol (III)

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add Activated Acid (II or IV), mix 1 h, drain</td>
</tr>
<tr>
<td>2</td>
<td>DCM wash: Fill (6 units), mix 30 s, drain</td>
</tr>
<tr>
<td>3</td>
<td>DMF wash: Fill (6 units), mix 30 s, drain</td>
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Table 2.6  Terminal Pyrrole Activation Protocol (IV)

<table>
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<td>1</td>
<td>Py-COOH</td>
<td>0.186 g, 1.49 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.540 g, 1.42 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.75 mL, 4.31 mmol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Mix 5 min</td>
</tr>
</tbody>
</table>

† † † 1 unit = approximately 1.15 mL.
2.8.2 Fmoc-β-Ala-Chlorotrityl Resin

![Fmoc-β-Ala-Chlorotrityl Resin](image)

2-Chlorotrityl chloride resin (0.5g, 0.5 mmol) was dissolved in anhydrous DCM (5 mL). Separately, Fmoc-β-Ala-OH (0.156 g, 0.5 mmol) in anhydrous DCM (4 mL) and DIEA (0.257 g, 0.348 mL, 2 mmol) was stirred for 5 min. The Fmoc-β-Ala-OH solution was added to the resin mixture and shaken for 5 h. MeOH (2.5 mL) was added and the mixture was shaken for 30 min. The resin was filtered, washed with DCM and dried. Yield (0.655 g, 0.622 mmol g\(^{-1}\))

2.8.3 Synthesis of LLSP4 Using Fmoc-β-Ala-Chlorotrityl Resin

![LLSP4](image)

The polyamide LLSP4 was prepared with machine assisted protocols using Fmoc-β-Ala-chlorotrityl resin, prepared as described in Section 2.8.2, on a Quartet Symphony Peptide Synthesiser. The resin was dried under vacuum before use.
2.8.3.1 LLSP4 Procedure

Fmoc-β-Ala-chlorotrityl resin (0.655 g, 0.407 mmol) was weighed into a reaction vessel, washed and deprotected using Cleavage Protocol (I) (Table 2.7)‡‡‡. During step 4 of (I), Pyrrole Activation Protocol (II) (Table 2.8) was initiated. After mixing for 5 min, the activated pyrrole solution was added to the reaction vessel and Coupling Protocol (III) (Table 2.9) was commenced. Protocols (I) to (III) were then repeated sequentially, two more times. Addition of the fourth pyrrole ring was achieved using protocols (I), Terminal Pyrrole Activation Protocol (IV) (Table 2.10) and then (III). The resin was then washed twice with DCM (7 units, mix 30 s), twice with DMF (7 units, mix 30 s), twice with DCM (7 units, mix 30 s) and then dried for 5 min (N₂(g)). The resin was then transferred to a sample vial and added 0.6 mL acetic acid, 1.2 mL trifluoroethanol (TFE), and 4.2 mL DCM were added. The solution was then stirred gently for 1.5 h, filtered, and the resin washed with TFE:DCM (1:4, 6 mL). The filtrates were combined and the solvent evaporated under reduced pressure until ~2 mL remained. Water was added (5 mL) and the product was then lyophilised to yield a light brown solid. (71 mg, 27%)

Table 2.7 Cleavage Protocol (I)

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Units</th>
<th>Mix time</th>
<th>Drain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>3</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>3 min</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>17 min</td>
<td>On</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>7</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
</tbody>
</table>

‡‡‡ Note the machine assisted protocols are different from those used with WANG-OH resin.
### Table 2.8  Pyrrole Activation Protocol (II)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Py-COOH</td>
<td>0.230 g, 0.62 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.212 g, 0.56 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.31 mL, 1.78 mmol</td>
</tr>
<tr>
<td>5</td>
<td>Mix 5 min</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.9  Coupling Protocol (III)

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add Activated Acid (II or IV), mix 3.5 h, drain</td>
</tr>
<tr>
<td>2</td>
<td>DCM wash: Fill (7 units), mix 30 s, drain</td>
</tr>
<tr>
<td>3</td>
<td>DMF wash: Fill (7 units), mix 30 s, drain</td>
</tr>
</tbody>
</table>

### Table 2.10  Terminal Pyrrole Activation Protocol (IV)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Py-COOH</td>
<td>0.186 g, 1.49 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.540 g, 1.42 mmol</td>
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<tr>
<td>4</td>
<td>DIEA</td>
<td>0.75 mL, 4.31 mmol</td>
</tr>
<tr>
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<td>Mix 5 min</td>
<td></td>
</tr>
</tbody>
</table>

### 2.8.4 Synthesis of LLSP4-Pt Using Chlorotrityl Resin

The polyamide, LLSP4-Pt, was prepared with machine assisted protocols using Fmoc-β-Ala-Chlorotrityl resin, prepared as described in Section 2.8.2, on a Quartet Symphony Peptide Synthesiser. The resin was dried under vacuum before use.
2.8.4.1 LLSP4-Pt Procedure

Fmoc-β-Ala-chlorotrityl resin (0.364 g, 0.28 mmol) was weighed into a reaction vessel, washed and deprotected using Cleavage Protocol (I) (Table 2.11). During step 4 of (I), Pyrrole Activation Protocol (II) (Table 2.12) was initiated. After mixing for 5 min, the activated pyrrole solution was added to the reaction vessel and Coupling Protocol (III) (Table 2.13) was commenced. Protocols (I) to (III) were then repeated sequentially, three more times. The resin was then deprotected using (I) and during step 4 Linker Activation Protocol (IV) (Table 2.14) was commenced. The activated linker solution was then added to the reaction vessel after stirring for 5 min and (III) was started. Activated trans-DDP(V) (Table 2.15) was prepared in advance and added to the resin. TEA (0.273 mL, 1.96 mmol) was added to the suspension which was then mixed for 12 h in the dark and drained. The resin was then washed twice with DCM (7 units, mix 30 s), twice with DMF (7 units, mix 30 s), and twice with DCM (7 units, mix 30 s). The resin was then washed twice in brine, twice in brine and DCM (1:1), five times in water, twice with DMF (7 units, mix 30 s), and twice with DCM (7 units, mix 30 s). The resin was then dried for 5 min N$_2$(g). The resin was then transferred to a sample vial and added 0.6 mL acetic acid, 1.2 mL TFE, and 4.2 mL DCM. The solution was then stirred gently for 1.5 h, filtered, and the resin washed with TFE:DCM (1:4, 6 mL). The cleavage reaction was then repeated two more times yielding three separate samples in total. The samples were then evaporated under reduced pressure until ~2 mL remained in each. Water was added to each sample (5 mL) and the products were then lyophilised to yield light brown solids. Compound was found in fractions 2 and 3 (125 mg, 31%). $^1$H NMR 300 MHz, ($d_6$-DMSO) δ 10.77 (s, 1H), 9.95 (s, 1H), 9.88 (s, 1H), 9.84 (s, 1H), 8.01 (t, 1H, J = 5.7 Hz), 7.30 (s, 1H), 7.22 (s, 2H), 7.17 (s, 1H), 7.05 (s, 1H), 7.04 (s, 1H), 6.98 (s, 1H), 6.83 (s, 1H), 5.96 (bs, 2H), 5.78 (bs, 2H), 4.02 (s, 6H), 3.98 (s, 6H), 3.86 (s, 3H), 3.85 (s, 3H), 3.84 (s, 3H), 3.80 (s, 3H), 2.39 (t, 3H, J = 7.2 Hz), 1.89 (bs, 2H), 1.75 (bs, 2H), 1.62 (bs, 2H), 1.29 (bs, 4H); ESI-MS: m/z: calcd for C$_{33}$H$_{55}$Cl$_2$N$_{15}$O$_7$Pt$_2$: 1234.95; found: 1233.3 [M-H]$^-$. Anal. Calcd. for C$_{39}$H$_{69}$Cl$_4$N$_{15}$O$_{14}$Pt$_2$ (LLSP4-Pt.H$_2$O .3CH$_3$CO$_2$H): C, 31.14; H, 4.62; N, 13.97 %. Found: C, 31.06; H, 4.53; N, 13.80 %.

Note the machine assisted protocols are different from those used with WANG-OH resin.
### Table 2.11 Cleavage Protocol (I)

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Units</th>
<th>Mix time</th>
<th>Drain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>3</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>3 min</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>piperidine/DMF</td>
<td>6</td>
<td>17 min</td>
<td>On</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>7</td>
<td>DMF</td>
<td>6</td>
<td>30 s</td>
<td>On</td>
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</table>

### Table 2.12 Pyrrole Activation Protocol (II)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Py-COOH</td>
<td>0.230 g, 0.62 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.212 g, 0.56 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.31 mL, 1.78 mmol</td>
</tr>
<tr>
<td>5</td>
<td>Mix 5 min</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2.13 Coupling Protocol (III)

<table>
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<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add Activated Acid (II or IV), mix 3.5 h, drain</td>
</tr>
<tr>
<td>2</td>
<td>DCM wash: Fill (7 units), mix 30 s, drain</td>
</tr>
<tr>
<td>3</td>
<td>DMF wash: Fill (7 units), mix 30 s, drain</td>
</tr>
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</table>

### Table 2.14 Linker Activation Protocol (IV)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>di-Fmoc-Lysine-OH</td>
<td>0.366 g, 0.62 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.212 g, 0.56 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.31 mL, 1.78 mmol</td>
</tr>
<tr>
<td>5</td>
<td>Mix 5 min</td>
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</tr>
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</table>

### Table 2.15 trans-DDP Activation Protocol (V)

<table>
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<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>trans-DDP</td>
<td>0.420 g, 1.40 mmol</td>
</tr>
<tr>
<td>2</td>
<td>AgNO₃</td>
<td>0.214 g, 1.26 mmol</td>
</tr>
<tr>
<td>3</td>
<td>DMF</td>
<td>5 mL</td>
</tr>
<tr>
<td>4</td>
<td>Stir Overnight (N₂/g/dark)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Filter</td>
<td></td>
</tr>
</tbody>
</table>
2.9 Solid Phase Discussion

Polyamide synthesis was initially attempted via solution chemistry but problems such as low yields and purity were encountered. This method was also limited to the number of rings that could be coupled successively; after three rings no further coupling could be achieved. Solid phase synthesis was employed, which produced pure compounds at high yields. The first compound made was the precursor LLSP4-DPA which had no platinum complex attached in order to simplify initial synthesis and confirm the coupling method conditions. The polyamide LLSP4-DPA was made with WANG-OH resin on a Quartet Symphony Peptide synthesiser as shown in Scheme 2.3.

![Scheme 2.3](image-url)

**Scheme 2.3** Solid phase synthesis of LLSP4-DPA on WANG-OH resin: (i) 20% Fmoc-Py acid (inset), HBTU, DIEA; (v) 20% piperidine/DMF; (vi) Fmoc-Py acid (inset), HBTU, DIEA; (vii) 20% piperidine/DMF; (viii) Py-acid (inset), HBTU, DIEA; (ix) N,N’-dimethylaminopropylamine, 55 °C.
The machine assisted protocols implemented for the synthesis of LLSP4-DPA used short coupling times (1 h), as described in previous methods, with the addition cycle (including washing and deprotection) taking approximately 2 h per residue.\textsuperscript{155} Cleavage of the polyamide from the solid support was achieved by aminolysis using 3-dimethylaminopropylamine. Rotary evaporation of the excess amine was not effective as the compound appeared as an oily residue and had the characteristic smell of the cleaving agent present even after several hours under reduced pressure (<1 torr). Precipitation of the polyamide in water did aid in the removal of some of the amine, but impurities were still found. Positive ion ESI mass spectrometry of the sample found the parent ion at 647 \( m/z \), but also found the cleaving agent at 103.2 \( m/z \) with a 63% relative abundance. Attempts to purify the polyamide via HPLC methods were unsuccessful, even reported procedures claimed to have residual amine impurity after HPLC purification. \(^1\)H NMR of LLSP4-DPA also showed the presence of the 3-dimethylaminopropylamine impurity with several extra peaks in the aliphatic region (Figure 2.24).

\textbf{Figure 2.24} \(^1\)H NMR spectrum of LLSP4-DPA, 300 MHz, 35 °C, \( d_6 \)-DMSO. The impurities are denoted by the arrows.
The impurities encountered when using the WANG-OH resin led to the search for a more suitable resin for the application. WANG resins also have inherent problems in the first few coupling cycles; if two proline groups are coupled in the first two cycles diketopiperazine adducts are formed which effectively terminate the peptide chain by capping the end prematurely. It is unknown if the formation of these diketopiperazine adducts would also apply to the polyamides in this project.

The next resin used was chlorotrityl (Figure 2.25), an acid labile support which produces a free acid C-terminus when cleaved. Another advantage of this resin is its ability to leave protecting groups intact, an application not used here but possibly useful in future work when coupling multiple metal complexes to the one polyamide.

![Figure 2.25](image)

**Figure 2.25** Chlorotrityl resin is prepared by condensation with Fmoc-β-Ala-OH to produce the protected resin ready for monomer additions.

Chlorotrityl resin is prepared by first adding a β-alanine group which prevents the possible formation of diketopiperazine adducts. The resin was dried under vacuum before use as the condensation reaction of Fmoc-β-Ala-OH and chlorotrityl resin is water sensitive. The DCM used in the reaction was also dried using a basic aluminium oxide column. After shaking for 5 h, MeOH was added to cap the unreacted chlorotrityl groups, which prevents coupling to an unsubstituted β-Ala site. It was important that the resin was shaken in MeOH for no longer than 30 min as the yield decreases. It is suspected that longer capping times may cause cleavage of Fmoc-β-Ala-OH from the resin. The resin was then washed, dried under vacuum and then weighed to calculate the molar loading capacity (moles per gram) of the resin.
The first compound made using chlorotrityl resin was the polyamide LLSP4, made in order to test the coupling success prior to the attachment of trans-DDP. Reactions times for the coupling of pyrrole rings were increased to 3.5 h. This was done in an effort to help improve purity of the product by reducing the amount of unreacted amine that may be present if insufficient reaction times were used. To determine if 3.5 h was sufficient time to couple the pyrrole rings, a ninhydrin test was performed to identify primary amines. It was observed under a microscope that before 3.5 h some of the resin still remained unreacted (blue), while after 3.5 h the resin was all reacted (red). After the coupling of four pyrrole rings the polyamide was cleaved in acetic acid, TFE and DCM, and the resin then filtered. Resin cleavage was repeated two more times and the three resultant fractions were lyophilised. The first fraction recovered was analysed by NMR and was shown to contain many impurities. The second and third fractions afforded much higher yields and were shown to contain almost no impurities besides residual solvent as shown in the $^1$H NMR (Figure 2.26). On average, the yields obtained for the second and third fractions were considerably higher than those for fraction one. Analysis by positive ion ESI-MS showed an [M+H] ion at 563.3 m/z, supporting the successful synthesis of the polyamide.

![Figure 2.26](image)

**Figure 2.26** $^1$H NMR spectrum of LLSP4, 300 MHz, 35 °C, $d_6$-DMSO.
Analysis of the $^1$H NMR spectrum of LLSP4 also indicates that the polyamide was made successfully. The amide proton resonances are expected to be the furthest downfield as commonly seen in polyamides. The resonances at 9.88, 9.84 and 9.77 ppm, are designated as the three amides that are found adjacent to two pyrrole rings and appear as singlets as they have no neighbouring protons. The proton resonance at 8.00 ppm is assigned as the amide adjacent the $\beta$-alanine group and appears as a triplet as it has two neighbouring protons. The resonances between 7.22 and 6.05 ppm are all assigned as aromatic protons from the pyrrole rings. The four singlets at 3.88, 3.85, 3.84 and 3.79 integrate to three protons each and are assigned as the N-methyl groups on the pyrrole rings. The resonances at 3.36 and 2.42 ppm each integrate to 2 protons and are designated as the $\beta$-alanine aliphatic groups.

The final polyamide made was the dinuclear platinum complex LLSP4-Pt. The method used was similar to that of LLSP4 with changes to some of the conditions. The equivalents of pyrrole monomer added to the resin were increased from 1.3 to 2.2. This was done in an effort to avoid the possibility of side products by maximising coupling yields. Synthesis began with the coupling of four pyrrole monomers to the resin. Next, di-Fmoc-lysine-OH, a six carbon chain with Fmoc protected amines at the two and six positions, was coupled to the polyamide chain. After attachment of this linker and deprotection of the amines, activated trans-DDP was added to the reaction vessel and the suspension allowed to mix for 12 h. Following platination the resin was washed with the standard DMF and DCM protocols but also included a brine wash and a brine/DCM wash in an attempt to ensure the complex was a chloride salt when freed from the resin. DCM was included in the second brine wash as it aided in the mixing of the hydrophobic beads with the aqueous solution. To ensure all the salt was removed, the resin was washed several times with water (5 rinses) followed by DMF and DCM washes. The resin was then dried and cleaved with acetic acid as done previously. The compound was collected in fractions and each reduced in volume. Water was then added and the samples were lyophilised to yield a fluffy, cream coloured compound that was easily manageable.
CHAPTER 2: POLYAMIDE SYNTHESIS

Characterisation of LLSP4-Pt by positive ion ESI-MS showed the M-H ion at 1233.3 \textit{m/z}. The [M+H] ion which should appear at 411.98 \textit{m/z} was not present although a nearby peak at 413.40 \textit{m/z} was found. The M-H ion provided a strong indication that the platinum groups were successfully coordinated. The presence of the two platinum nuclei in LLSP4-Pt were then confirmed by $^{195}$Pt NMR. The spectrum showed a peak at -2426 ppm with a shoulder at -2428 ppm (Figure 2.27).

![Figure 2.27 $^{195}$Pt NMR spectrum of LLSP4-Pt, 400 MHz, 35 °C, $d_6$-DMSO.](image)

$^1$H NMR of LLSP4-Pt showed the product was successfully synthesised with some minor impurities present in the spectrum (Figure 2.28). Assignments of the peaks were as follows. The resonances at 10.77, 9.95, 9.88, 9.84 and 8.01 ppm, were assigned as amide protons as they characteristically appear between 8.00 and 11.00 ppm. These resonances also integrated to 1 proton each. The triplet at 8.01 ppm was assigned as the amide adjacent the $\beta$-alanine group as it is the only amide next to a methylene group. The resonances at 7.30, 7.22, 7.17, 7.05, 7.04, 6.98, and 6.83 ppm, are designated as pyrrole hydrogens, integrating to 1 proton each. The broad singlets integrating to 2 protons each at 5.96 and 5.78 ppm are identified as NH$_2$ groups as primary amines have a chemical shift typically found between 5.0 and 6.0 ppm. The resonances at 4.02 and 3.98 ppm are assigned as NH$_3$ protons. Each peak integrates to 6 protons suggesting there are two amines present per resonance. The methyl groups are found at 3.86, 3.85, 3.84 and 3.80 ppm, appearing as broad singlets that integrate to 3 protons each. The remaining peaks are the aliphatic proton resonances from the di-Fmoc-lysine-OH linker and the $\beta$-alanine group at 2.39, 1.89, 1.75, 1.62 and 1.29 ppm. It was impossible to assign each resonance to specific protons as the individual subunits share similar chemical environments making their resonances indistinguishable.
Elemental analysis of LLSP4-Pt identified some important properties of the recovered sample. Initial calculations included the molecule and two chloride counter ions; C, 30.35, H, 4.24, N, 16.09%. The results obtained did not agree with the calculated percentages and deviated as far as 2.29%. Addition of one water of hydration and three acetic acid molecules to the calculation did match the results although this did not agree with the integrals in the $^1$H NMR (most likely due to deuterium exchange with acetic acid protons). Attempts to remove the solvent contaminants by repeated evaporation in ethanol (EtOH) and lyophilisation were unsuccessful, as evident in the NMR for LLSP4-Pt. Table 2.16 shows the summarised $^1$H NMR data for the polyamides synthesised with solid phase chemistry.
### Table 2.16  
$^1$H NMR Data for Solid Phase Polyamides.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proton Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amide</td>
</tr>
<tr>
<td>LLSP4-DPA</td>
<td></td>
</tr>
<tr>
<td>9.90 (s, 1H)</td>
<td>7.23 (d, 2H, $J = 1.8$ Hz)</td>
</tr>
<tr>
<td>9.85 (s, 1H)</td>
<td>7.19 (d, 1H, $J = 1.8$ Hz)</td>
</tr>
<tr>
<td>9.81 (s, 1H)</td>
<td>7.05 (t, 2H, $J = 1.8$ Hz)</td>
</tr>
<tr>
<td>7.40 (t, 1H, $J = 5.4$ Hz)</td>
<td>6.94 (s, 1H)</td>
</tr>
<tr>
<td>7.85 (t, 1H, $J = 5.7$ Hz)</td>
<td>6.83 (d, 1H, $J = 1.8$ Hz)</td>
</tr>
<tr>
<td>LLSP4</td>
<td></td>
</tr>
<tr>
<td>9.88 (s, 1H)</td>
<td>7.22 (d, 1H, $J = 1.5$ Hz)</td>
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<td>9.84 (s, 1H)</td>
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<td>6.05 (dd, 1H, $J = 2.7 / 3.9$ Hz)</td>
</tr>
<tr>
<td>LLSP4-Pt</td>
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<tr>
<td>10.77 (s, 1H)</td>
<td>7.30 (s, 1H) 7.22 (s, 2H)</td>
</tr>
<tr>
<td>9.95 (s, 1H)</td>
<td>7.17 (s, 1H) 7.05 (s, 1H)</td>
</tr>
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<td>7.04 (s, 1H) 6.98 (s, 1H)</td>
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<td>6.83 (s, 1H)</td>
</tr>
<tr>
<td>8.01 (t, 1H, $J = 5.7$ Hz)</td>
<td>6.05 (dd, 1H, $J = 2.7 / 3.9$ Hz)</td>
</tr>
</tbody>
</table>

### 2.10 Conclusion

This chapter reports on the development of LLSP4-Pt, which began with solution phase techniques. Methyl 1-methyl-4-nitropyrole-2-carboxylate was made via esterification of 1-methyl-4-nitropyrole-2-carboxylate and used as a synthon for polyamide chain extension. Hydrogenation of methyl 1-methyl-4-nitropyrole-2-carboxylate produced an unstable amine which was then coupled to 1-methyl-2-pyrole carboxylic acid. These additions were repeated two more times to produce PyPyPyCO$_2$H, the synthesis of which encountered several problems. Low yields and purification issues were encountered with the intermediates involved greatly hindering the progression of the project. Attempts to couple a fourth pyrole-ring the polyamide was unsuccessful.
Development of the polyamides then progressed to solid phase chemistry. The Fmoc protected, pyrrole monomer Py5, required for solid phase synthesis, was made in high yield and purity following adaptation of several published literature methods. Acetylation of 1-methylpyrrole with trichloroacetyl chloride yielded Py1 which was then nitrated to produce the nitro species Py2. The trichloroacetyl group of Py2 was then substituted for a tert-butyl to make Py3. Hydrogenation of Py3 produced the free amine species which was then Fmoc protected to form Py4. Removal of the tert-butyl ester yielded the solid phase synthon Py5 which was characterised by $^1$H NMR techniques.

The precursor molecule LLSP4-DPA was made using machine assisted protocols on a peptide synthesiser with Fmoc-β-Ala-OH-WANG resin. Impure products from cleavage reactions for LLSP4-DPA were a result of aminolysis; the cleaving agent 3-dimethylaminopropylamine could not be separated from the product and persisted as a contaminant. Fmoc-β-Ala-chlorotrityl resin was therefore adopted an alternative to Fmoc-β-Ala-OH-WANG resin. Synthesis of the second precursor, LLSP4, using Fmoc-β-Ala-chlorotrityl resin, resulted in only slightly higher yields but with a much greater purity. LLSP4 was characterised by $^1$H NMR and mass spectrometry.

The target metallo-polyamide LLSP4-Pt was made using Fmoc-β-Ala-chlorotrityl resin with machine assisted protocols on a peptide synthesiser. Cleavage of the complex was accomplished by heating in TFE and acetic acid which afforded the product with only minor impurities present. The complex was characterised by NMR, ESI-MS, and elemental analysis, verifying synthesis of the compound was successful.
Chapter 3: Ruthenium Polyamide Synthesis

3.1 Introduction

The interactions between small synthetic molecules and DNA structure is an active area of research. In particular, transition metal complexes have gathered much interest because of their chemical inertness, cationic nature and easily modulated structure. Metal complexes also incorporate functionality with an aptitude for photophysical and redox properties making them interesting candidates as DNA binders.

The design and synthesis of ruthenium(II) polypyridyl complexes has advanced significantly over the past 20 years. They have been used to examine the tertiary structure of nucleic acids, act as artificial nucleases and luminescent probes, and investigate the electron transfer mediated by DNA. More recently, the development of a ruthenium/polyamide conjugates using solid phase synthesis have been investigated. These studies used carboxylated bipyridine ligands, from which the metal complex or free ligand was coupled to the resin bound polyamide. The complex made by Reedijk’s group, [Ru(terpy)(4-CO$_2$H-4'-Mebpy-Gly-L-His-L-LysCONH$_2$)][PF$_6$], has been shown to coordinatively bind to DNA with a preference for guanosine and cytosine bases. Barton and coworkers developed a series of ruthenium-peptide intercalators that serve as luminescent cross-linking probes for DNA.

Here we describe the development of the novel metallointercalator-peptide conjugate, LLSP4-Ru (Figure 3.1). The synthesis of the ruthenium complex and polyamide is reported and characterised by several techniques including NMR, ESI-MS, and fluorescence spectroscopy.
Figure 3.1 The ruthenium polyamide conjugate LLSP4-Ru.

3.2 Reagents

1,10-Phenanthroline 99% (phen), ruthenium trichloride trihydrate, 1,2-diaminoethane, sodium hydroxide, 8-aminoquinoline 98%, crotonaldehyde, benzotriazol-1-yloxy-tripyrrolidinophosphonium hexafluorophosphate 98% (PyBOP) were purchased from Sigma-Aldrich. Potassium bromide (KBr) 99% and selenium dioxide 99% were purchased from Alfa Aesar. Sodium hydrogen carbonate, sodium iodide and sodium carbonate were obtained from Ajax Chemicals. Dioxane and 4-amino butyric acid 97% were purchased from Fluka. Calf thymus (ct-DNA) (sonicated to ~2000 bp in length) was purchased from Gilbeo BRL. HNO₃ 69-70% was obtained from J T Baker. HCl was purchased from Lab Scan. H₂SO₄ 95-97% was obtained from Sharlau. Sodium sulphate 98% and tin(II) chloride anhydrous was purchased Riedel-de Haëg. All other reagents were of general laboratory grade or found in Section 2.2.

3.3 Instrumentation

Solid phase peptide synthesis, NMR spectrometry, and Positive ion ESI mass spectrometry were carried out using protocols found in Section 2.3. Fluorescence spectroscopy was conducted on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a peltier temperature controller. An excitation wavelength of 450 nm was used with medium voltage detector settings.
3.4 Experimental

3.4.1 1,10-Phenanthroline-5,6-dione (phendo)

Synthesis was adapted from the method of Yamada et al.\textsuperscript{179} 1,10-Phenanthroline (40 g, 0.22 mol) and KBr (40 g, 0.34 mol) was added a chilled mixture of conc. H\textsubscript{2}SO\textsubscript{4} (400 mL, conc.) and HNO\textsubscript{3} (200 mL, 70.0%) over 1 h. The mixture was then heated at reflux at 90 °C for 3 h. The solution was cooled and then neutralised using sodium hydrogen carbonate (NaHCO\textsubscript{3}) (1.5 kg, 18 mol). The solution was extracted 3 times with DCM (4 L) and dried over anhydrous Na\textsubscript{2}SO\textsubscript{4}. The solvent was removed under reduced pressure and the crude product recrystalised from MeOH (38.4 g, 83%). \textsuperscript{1}H NMR 300 MHz, (\textit{d}\textsubscript{6}-DMSO) δ 8.99 (bs, 2H, H4), 8.38 (bs, 2H, H2), 7.59 (dd, 2H, J = 4.7 / 7.6 Hz, H3).

3.4.2 Dipyrido[3,2-d:2'3'-f]quinoxaline (dpq)

Phendo (5 g, 23.7 mmol) was dissolved in absolute EtOH (700 mL), with heat and stirring. 1,2-diaminoethane (20 g, 333 mmol) in absolute EtOH (100 mL) was added drop wise to the refluxing phendo solution over a period of 1 h. The solution was heated at reflux for an additional hour, before activated charcoal was added and the solution heated at reflux for a further hour, then left to stir for 48 h. The solution was heated at reflux, filtered, and H\textsubscript{2}O
(100 mL) added before the EtOH was removed under vacuum. The cream coloured product was collected, washed with copious amounts of boiling H₂O and diethyl ether and then air-dried (3.65 g, 73%). **¹H NMR 300 MHz, (CD₃CN) δ 9.46 (dd, 2H, J = 1.8 / 8.2 Hz, H4), 9.19 (dd, 2H, J = 1.8 / 4.4 Hz, H2), 9.01 (s, 1H, H7), 7.84 (dd, 2H, J = 4.4 / 8.1 Hz, H3).

### 3.4.3 4-Methyl-1,10-phenanthroline (4-Me-phen)

Synthesis was adapted from the method of Belser *et al.* Crotonaldehyde (16.26 g, 0.23 mol) was added over 5 h to a stirred solution of 8-aminoquinoline (20 g, 0.14 mol) and sodium iodide (NaI) (0.2 g, 1.3 mmol) in H₂SO₄ (50.4 mL, 580 mmol) at 110 °C. The solution was then stirred for 1 h (110 °C) and the solution cooled to room temperature, poured into 1 M Na₂CO₃ (580 mL) and extracted with DCM (3 × 300 mL). The combined organic layers were extracted 12 M HCl (5 × 250 mL), the acidic solution neutralised (3 M NaOH and 1 M Na₂CO₃), and then extracted with DCM (3 × 250 mL). The solvent was removed under reduced pressure affording a light brown compound (5.37 g, 20%). **¹H NMR 300 MHz, (d₆-DMSO) δ 9.09 (dd, 1H, J = 1.8 / 4.3 Hz, H9), 8.94 (d, 1H, J = 4.4 Hz, H2), 8.48 (dd, 1H, J = 1.8 / 8.1 Hz, H7), 8.14 (d, 1H, J = 9.1 Hz, H6), 8.01 (d, 1H, J = 9.1 Hz, H5), 7.75 (dd, 1H, J = 4.3 / 8.1 Hz, H8), 7.61 (dd, 1H, J = 0.9 / 4.6 Hz, H3), 2.78 (s, 3H).
3.4.4 4-Carboxy-1,10-phenanthroline (4-CO$_2$H-phen)

![4-Carboxy-1,10-phenanthroline](image)

Synthesis was adapted from the method of Yanagida et al.$^{181}$ 4-Me-phen (2.0 g, 10.4 mmol) was dissolved in dioxane (43 mL, 44.4 g, 0.5 mol) containing 4% water (1.8 mL) and selenium dioxide (1.8g, 16.2 mmol) was added and the mixture left to reflux for 2 h. The mixture was then filtered and the solvent reduced. HNO$_3$ (10 mL) was added, the solution refluxed for 3 h and the reaction quenched on ice. The light yellow product was then collected and washed with copious amounts of water (200 mL) (1.37 g, 59%).$^1$H NMR 300 MHz, (d$_6$-DMSO) $\delta$ 9.38 (d, 1H, $J = 4.6$ Hz, H3), 9.29 (dd, 1H, $J = 1.4 / 5.2$, H9), 9.20 (dd, 1H, $J = 1.4 / 8.3$ Hz, H2), 8.92 (d, 1H, $J = 9.3$ Hz, H5), 8.39 (d, 1H, $J = 3.2$ Hz, H7), 8.37 (d, 1H, $J = 1.5$ Hz, H6), 8.28 (dd, 1H, $J = 5.2 / 8.2$ Hz, H8).

3.4.5 [Ru(dpq)$_2$Cl$_2$] Method A

![Ru(dpq)$_2$Cl$_2$](image)

Synthesis was adapted from the method of Anderson et al.$^{182}$ RuCl$_3$.3H$_2$O (1.71 g, 7 mmol) was dissolved in DMF (65 mL), added dpq (3.25 g, 14 mmol), and heated at reflux for 6 h. The DMF was evaporated under reduced pressure until ~10 mL remained. Acetone was added (500 mL) and the solution left at 4 °C overnight. The precipitate was collected and dissolved in EtOH:H$_2$O 1:1 (250 mL), heated at reflux for 1 h and then filtered. The filtrate was added lithium chloride and the EtOH evaporated under reduced pressure. The resultant precipitate was collected and air-dried (2.90 g, 65%).
3.4.6 [Ru(dpq)$_2$Cl$_2$] Method B

Synthesis was adapted from the method of Hua et al.$^{183}$ RuCl$_3$.3H$_2$O (0.4 g, 1.7 mmol) was dissolved in EtOH /H$_2$O (3:2, 75 mL) and the mixture heated at reflux until the solution turned a deep blue (3.5 h). Dpq (1.0 g, 4.3 mmol), dissolved in EtOH /HCl (14:1, 22 mL) was added and the resulting solution heated at reflux overnight. The solution was reduced to 50 mL and filtered to collect the solids that had precipitated. The brown solids were washed with acetone (10 mL) and diethyl ether (50 mL), before resuspension in HCl (3 M, 250 mL) and tin(II) chloride (SnCl$_2$).2H$_2$O (70 mg, 0.32 mmol) was added. The solution was heated at reflux (under N$_2$(g)) for 2.5 h before the solid [Ru(dpq)$_2$Cl$_2$] was collected under vacuum, washed with copious amounts of cold H$_2$O and air-dried. The crude product was then purified via column chromatography (silica, 2% KNO$_3$, ACN) (0.58 g, 54%).

3.4.7 Synthesis of LLSP4-(4-CO$_2$H-phen)

The polyamide, LLSP4-(4-CO$_2$H-phen), was prepared with machine assisted protocols using Fmoc-$\beta$-ala-chlorotrityl resin, prepared as described in Section 2.8.2, on a Quartet Symphony Peptide Synthesiser. The resin was dried under vacuum before use.
3.4.7.1 LLSP4-(4-CO$_2$H-phen) Procedure

Fmoc-$\beta$-Ala-chlorotrityl resin (0.364 g, 0.28 mmol) was weighed into a reaction vessel, washed and deprotected using Cleavage Protocol (I) (Resin Preparation) (Table 3.1). During step 4 of (I), Pyrrole Activation Protocol (II) (Table 3.2) was initiated. After mixing for 5 min, the activated pyrrole solution was added to the reaction vessel and Coupling Protocol (III) (Table 3.3) was commenced. Protocols (I) to (III) were then repeated sequentially, three more times. The resin was then deprotected using (I) and during step 4 (I), Linker Activation Protocol (IV) (Table 3.4) was commenced. The activated linker solution was then added to the reaction vessel after stirring for 5 min and Coupling Protocol (III) was followed. 4-CO$_2$H-phen Activation Protocol (V) (Table 3.5) was then employed, added to the reaction vessel, and (III) was commenced. The resin was then dried for 5 min (under N$_2$(g)) and transferred to a sample vial. The resin was then transferred to a sample vial and added 0.6 mL acetic acid, 1.2 mL TFE, and 4.2 mL DCM. The solution was then stirred gently for 1.5 h, filtered, and the resin washed with TFE:DCM (1:4, 6 mL). The cleavage reaction was then repeated two more times yielding three separate samples in total. The samples were then evaporated under reduced pressure until ~2 mL remained in each. Water was added to each sample (5 mL) and the products were then lyophilised to yield a pale yellow compound (125 mg, 31%). $^1$H NMR 300 MHz, ($d_6$-DMSO) $\delta$ 9.87 (bs, 1H), 9.85 (bs, 1H), 9.83 (bs, 2H), 9.14 (d, 1H, $J = 4.4$ Hz), 9.10 (dd, 1H, $J = 1.7 / 4.3$ Hz), 8.84 (t, 1H, $J = 5.6$ Hz), 8.48 (dd, 1H, $J = 1.7 / 8.1$ Hz), 8.12 (d, 1H, $J = 9.1$ Hz), 8.01 (d, 1H, $J = 9.2$ Hz), 7.95 (t, 1H, $J = 5.6$ Hz), 7.78 (m, 1H, $J = 4.29 / 8.07$ Hz), 7.76 (d, 1H, $J = 4.4$ Hz), 7.21 (bs, 2H), 7.16 (bs, 2H), 7.04 (m, 2H, $J = 1.7 / 3.7$ Hz), 6.90 (d, 1H, $J = 1.7$ Hz), 6.84 (d, 1H, $J = 1.8$ Hz), 3.84 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.78 (s, 3H), 3.43 (t, 2H, $J = 6.6$ Hz), 3.36 (t, 2H, $J = 6.0$ Hz), 2.46 (t, 2H, $J = 7.2$ Hz), 2.39 (t, 2H, $J = 7.5$ Hz), 1.91 (t, 2H, $J = 7.2$ Hz). ESI-MS: $m/z$: calcld for C$_{44}$H$_{44}$N$_{12}$O$_8$: 868.9; found: 869.3 [M+H]$^+$. 
### Table 3.1 Cleavage Protocol (I)

<table>
<thead>
<tr>
<th>Step</th>
<th>Solvent</th>
<th>Units</th>
<th>Mix time</th>
<th>Drain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DCM</td>
<td>7</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>7</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>3</td>
<td>piperidine/DMF</td>
<td>7</td>
<td>3 min</td>
<td>On</td>
</tr>
<tr>
<td>4</td>
<td>piperidine/DMF</td>
<td>7</td>
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<td>On</td>
</tr>
<tr>
<td>5</td>
<td>DMF</td>
<td>7</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>6</td>
<td>DCM</td>
<td>7</td>
<td>30 s</td>
<td>On</td>
</tr>
<tr>
<td>7</td>
<td>DMF</td>
<td>7</td>
<td>30 s</td>
<td>On</td>
</tr>
</tbody>
</table>

### Table 3.2 Pyrrole Activation Protocol (II)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Py-COOH</td>
<td>0.230 g, 0.62 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.212 g, 0.56 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.31 mL, 1.78 mmol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Mix 5 min</td>
</tr>
</tbody>
</table>

### Table 3.3 Coupling Protocol (III)

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Add Activated Acid (II or IV), mix 3.5 h, drain</td>
</tr>
<tr>
<td>2</td>
<td>DCM wash: Fill (7 units), mix 30 s, drain</td>
</tr>
<tr>
<td>3</td>
<td>DMF wash: Fill (7 units), mix 30 s, drain</td>
</tr>
</tbody>
</table>

### Table 3.4 Linker Activation Protocol (IV)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-amino-butyric acid</td>
<td>0.20 g, 0.62 mmol</td>
</tr>
<tr>
<td>2</td>
<td>DMF</td>
<td>3 mL</td>
</tr>
<tr>
<td>3</td>
<td>HBTU</td>
<td>0.212 g, 0.56 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.31 mL, 1.78 mmol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Mix 5 min</td>
</tr>
</tbody>
</table>

### Table 3.5 4-CO$_2$H-phen Activation Protocol (V)

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-CO$_2$H-phen</td>
<td>0.695 g, 3.01 mmol</td>
</tr>
<tr>
<td>2</td>
<td>NMP</td>
<td>7 mL</td>
</tr>
<tr>
<td>3</td>
<td>PyBOP</td>
<td>2.8 g, 4.0 mmol</td>
</tr>
<tr>
<td>4</td>
<td>DIEA</td>
<td>0.93 mL, 5.3 mmol</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Mix 5 min</td>
</tr>
</tbody>
</table>
3.4.8 LLSP4-Ru

[Ru(dpq)₂Cl₂] (25 mg, 0.04 mmol) and LLSP4-(4-CO₂H-phen) (17 mg, 0.02 mmol) in 10 mL EtOH was heated at reflux for 16 h. The solution was cooled (4 °C) and the red solid collected and washed with cold EtOH (25 mg, 83%). ¹H NMR 300 MHz, (d₆-DMSO) δ 9.87 (bs, 1H), 9.86 (bs, 1H), 9.83 (bs, 2H), 9.52 (d, 2H, J = 8.3 Hz), 9.36 (bs, 4H), 9.14 (d, 1H, J = 4.2 Hz), 9.10 (d, 1H, J = 2.8 Hz), 8.85 (t, 1H, J = 5.4 Hz), 8.47 (d, 1H, J = 8.3 Hz), 8.45 (bs, 2H), 8.35 (m, 4H), 8.21 (d, 2H, J = 5.6 Hz), 8.17 (d, 2H, J = 5.0 Hz), 8.11 (d, 1H, J = 9.6 Hz), 8.01 (d, 1H, J = 9.1 Hz), 7.95 (t, 1H, J = 5.5 Hz), 7.79 (d, 1H, J = 4.2 Hz), 7.76 (d, 1H, J = 4.1 Hz), 7.21 (bs, 2H), 7.16 (bs, 2H), 7.03 (bs, 2H), 6.90 (bs, 1H), 6.84 (bs, 1H), 3.83 (bs, 9H), 3.79 (s, 3H), 3.40 (t, 2H), 3.35 (t, 2H), 2.39 (t, 2H, J = 7.5 Hz), 1.91 (t, 2H, J = 7.3 Hz).

3.4.9 Fluorescence DNA Binding

The fluorescence emission of LLSP4-Ru (0.177 μM) in 10 mM phosphate buffer was measured between 500 and 700 nm using an excitation wavelength of 450 nm. The fluorescence emission of the complex was then measured in the presence of ct-DNA (8.33 μM). The data was converted to ASCII format and analysed using Graph Pad Prism® version 4.03.
3.5 Discussion

3.5.1 Ligand and Metal Complex Synthesis

The synthesis of the dpq ligand was accomplished in two steps from the starting material phen. The addition of the acid mixture to phen was done with the solution chilled and added dropwise, resulting in higher yielding reactions. Neutralisation was afforded by adding NaHCO$_3$, the end point signalled by the disappearance of the effervescence. Subsequent extraction from the aqueous solution required large amounts of DCM to recover all the phendo, which was evaporated to yield the bright yellow compound. The $^1$H NMR spectrum of phendo is shown in Figure 3.2. Two broad peaks can be seen at 8.99 and 8.38 ppm, while a double doublet is seen at 7.59 ppm. The assignment of these resonances have been reported previously and are shown in Figure 3.2.$^{184}$

![Figure 3.2](image-url) $^1$H NMR spectrum of phendo, 300 MHz, 35 °C, d$_6$-DMSO.

The next step in ligand synthesis was the production of dpq via condensation with 1,2-diaminoethane. As the reaction is driven by the formation of water, it was important that freshly distilled 1,2-diaminoethane be used in addition to absolute EtOH. After heating at
reflux and stirring in activated charcoal for 48 h, the solution was heated back to reflux prior to filtration to release compound that would have otherwise remained adhered to the porous activated charcoal. Washing with large amounts of boiling water is necessary as it removes most of the impurities, while dpq remains insoluble. The $^1$H NMR spectrum of dpq is shown in Figure 3.3. The spectra shows the expected 4 aromatic resonances; a double doublet at 9.46, 9.19 and 7.84 ppm, and a singlet at 9.01 ppm. These assignments have been reported previously and are shown in Figure 3.3.$^{184}$

![Figure 3.3](image)

$^1$H NMR spectrum of dpq, 300 MHz, 25 °C, CD$_3$CN. The solvent impurities are denoted by the arrows.

The synthesis of 4-Me-phen was achieved via condensation of crotonaldehyde and 8-aminoquinoline using the method of Belser et al.$^{180}$ The extraction and neutralisation procedures of this method were seen to be work intensive; neutralising 1 L of 12 M HCl required a significant amount of a 3 M NaOH / 1 M Na$_2$CO$_3$ solution which then had to be extracted with 750 mL DCM. The yield reported (51%) by Belser et al. was not reproducible and the highest recovery of product obtained was 20%. These low yields and costly starting materials made it a very expensive procedure. The $^1$H NMR of 4-Me-phen is shown in Figure 3.4. The expected seven aromatic phen protons are seen in the spectra. There are four double doublets resonances at 9.09, 8.48, 7.75, and 7.61 ppm, while three doublets are seen at 8.94, 8.14, and 8.01 ppm. A good indication that the compound was made was the presence of the
methyl group which appears at 2.78 ppm and integrates to three protons. The assignments for 4-Me-phen have been reported previously by Lutun et al. and are shown in Figure 3.4. 

The oxidation of 4-Me-phen to the carboxylic acid (4-CO$_2$H-phen) was carried out through the synthesis of an aldehyde intermediate. The initial step involved heating at reflux in dioxane with selenium dioxide. The aldehyde was not filtered using celite (as described in the literature) as this resulted in a large decrease in yield due to product precipitation onto the celite bed. Instead, the solution was sonicated, brought back to reflux, and quickly filtered using a buchner funnel. The aldehyde was then dissolved in 10 mL HNO$_3$, ~5 times more than what is suggested by Yanagida et al. as this aided in dissolution of the compound and increased the yield significantly. The $^1$H NMR spectrum of 4-CO$_2$H-phen is shown in Figure 3.4 and assigned as previously reported. Seven aromatic protons are present; 4 doublets at 9.38, 8.92, 8.39, 8.37 ppm, and 3 double doublets at 9.29, 9.20, and 8.28 ppm. The broad carboxylic acid resonance can be seen at 5.25 ppm which disappeared in the presence of D$_2$O. The $^1$H NMR data obtained for ligand synthesis is summarised in Table 3.6.

![Figure 3.4](image-url) $^1$H NMR spectrum of 4-Me-phen, 300 MHz, 35 °C, $d_6$-DMSO.
Chapter 3: Ruthenium Polyamide Synthesis

Figure 3.5  $^1$H NMR spectrum of 4-CO$_2$H-phen, 300 MHz, 35 °C, $d_6$-DMSO.

Table 3.6 $^1$H NMR of the Ligands Synthesised.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proton Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aromatic</td>
</tr>
<tr>
<td>phendo</td>
<td>8.99 (bs, 2H, H4)</td>
</tr>
<tr>
<td></td>
<td>8.38 (bs, 2H, H2)</td>
</tr>
<tr>
<td></td>
<td>7.59 (dd, 2H, J = 4.7 / 7.6 Hz, H3)</td>
</tr>
<tr>
<td>dpq</td>
<td>9.46 (dd, 2H, J = 1.8 / 8.2 Hz, H4)</td>
</tr>
<tr>
<td></td>
<td>9.19 (dd, 2H, J = 1.8 / 4.4 Hz, H2)</td>
</tr>
<tr>
<td>4-Me-phen</td>
<td>9.09 (dd, 1H, J = 1.8 / 4.3 Hz, H9)</td>
</tr>
<tr>
<td></td>
<td>8.94 (d, 1H, J = 4.4 Hz, H2)</td>
</tr>
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<td>8.48 (dd, 1H, J = 1.8 / 8.1 Hz, H7)</td>
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<td>8.14 (d, 1H, J = 9.1 Hz, H6)</td>
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<td>7.75 (dd, 1H, J = 4.3 / 8.1 Hz, H8)</td>
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<td>7.61 (dd, 1H, J = 0.9 / 4.6 Hz, H3)</td>
</tr>
<tr>
<td>4-CO$_2$H-phen</td>
<td>9.38 (d, 1H, J = 4.6 Hz, H3)</td>
</tr>
<tr>
<td></td>
<td>9.29 (dd, 1H, J = 1.4 / 5.2, H9)</td>
</tr>
<tr>
<td></td>
<td>9.20 (dd, 1H, J = 1.4 / 8.3 Hz, H2)</td>
</tr>
<tr>
<td></td>
<td>8.92 (d, 1H, J = 9.3 Hz, H5)</td>
</tr>
<tr>
<td></td>
<td>8.39 (d, 1H, J = 3.2 Hz, H7)</td>
</tr>
<tr>
<td></td>
<td>8.37 (d, 1H, J = 1.5 Hz, H6)</td>
</tr>
<tr>
<td></td>
<td>8.28 (dd, 1H, J = 5.2 / 8.2 Hz, H8)</td>
</tr>
</tbody>
</table>
The synthesis of the \([\text{Ru(dpq)}_2\text{Cl}_2]\) precursor was achieved using two literature methods with varying results. The first of these techniques was the “ruthenium blue” method (Method A) of Anderson \textit{et al.}\textsuperscript{182}, the name being derived from the formation of the blue aqua species. The initial reflux in water/EtOH was carried out for 3 h, or until the solution turned a deep blue colour, to which dpq was then added. The \(\text{SnCl}_2\) reduction of ruthenium(III) to ruthenium(II) initially failed, it was later discovered to be due to the pre-oxidation of \(\text{SnCl}_2\). In subsequent reactions the \(\text{SnCl}_2\) was used immediately after weighing to avoid this problem, and the reaction carried out under nitrogen. The crude product showed many impurities when analysed by TLC, and column chromatography was necessary for purification.

The second method used was that of Hua \textit{et al.}\textsuperscript{183} (Method B) which began with refluxing \(\text{RuCl}_3.2\text{H}_2\text{O}\) with dpq for 6 h. Precipitation of the \textit{bis} species in acetone aided in the removal of unreacted \(\text{RuCl}_3\), while the following reflux in EtOH/water removed any \textit{tris} complex that may have formed. This method was the preferred as it resulted higher yielding products and with greater purity than Method A.

3.5.2 LLSP4-Ru Synthesis

Synthesis of the precursor molecule LLSP4-(4-CO\textsubscript{2}H-phen) was conducted using the methods developed in Chapter 2 for solid phase synthesis with chlorotrityl resin. Four pyrrole rings were coupled onto the resin followed by a butyric acid linker with an Fmoc protected amine. The next step was the addition of the 4-CO\textsubscript{2}H-phen ligand, which was attempted in several ways. HBTU was the coupling agent used in the first attempt but it did not yield any product and this was most likely due to non-compatibility of the acid. The next coupling agent chosen was PyBOP, used by Karidi \textit{at al.}\textsuperscript{177} for the analogous compound 4-carboxy-4-methyl-2,2'-bipyridine (4-CO\textsubscript{2}H-4'-Mebpy). The most noticeable difference was the 10.75 fold excess of 4-CO\textsubscript{2}H-phen required as used in Karidi’s reactions compared to the 2.2 fold excess of the pyrrole monomer necessary for standard coupling reactions used thus far. This in turn required a significant amount of PyBOP to activate (2.8 g) which in the end only
yielded 31% product despite the large excess. Cleavage of the product from the resin was initially done in one large fraction which resulted in extremely low yields (<5%) and impure product. When cleavage was performed and collected in several smaller fractions a higher yield and purity was obtained; fraction one was mainly impurities and discarded while fractions two and three were found to be product and were therefore combined.

LLSP4-(4-CO$_2$H-phen) was characterised by mass spectrometry and NMR spectroscopy. The ESI-MS of LLSP4-(4-CO$_2$H-phen) shows the parent [M+H] ion at 869.3 m/z which corresponds to the target compound. The $^1$H NMR for LLSP4-(4-CO$_2$H-phen) is shown in Figure 3.6.

**Figure 3.6** $^1$H NMR spectrum of LLSP4-(4-CO$_2$H-phen), 300 MHz, 35 °C, $d_6$-DMSO. The impurities are denoted by the arrows.

Due to the inclusion of a phen group in the spectra it was not possible to assign or distinguish the amide protons from the standard aromatic protons. The combined number of amide and aromatic protons (21) are seen in the spectra as determined by the integrals. The four pyrrole methyl groups appear at 3.84, 3.83, 3.82 and 3.78 ppm and confirm the presence of the correct number of polyamide rings. The remaining aliphatic protons were identified at 3.43, 3.36, 2.46, 2.39, and 1.91 ppm via integration. Impurities in the aliphatic region are also seen
and are believed to be low molecular weight residues that were cleaved from the resin. As mentioned earlier, many impurities are present in fraction one. It is believed that some of these impurities carry across to subsequent fractions and that collection of smaller fractions may help improve the purity of the end product in future experiments.

Efforts were initially made to synthesise LLSP4-Ru completely whilst the polyamide remained resin bound. This was attempted by refluxing LLSP4-(4-CO$_2$H-phen) with the bis-ruthenium complex, but it did not yield the desired product. It is hypothesised that the resin is unstable at high temperatures experienced during reflux and degradation of the product occurs.

The synthesis of the target ruthenium polyamide LLSP4-Ru was accomplished by heating at reflux [Ru(dpq)$_2$Cl$_2$] in solution with LLSP4-(4-CO$_2$H-phen). Optimisation of this reaction by varying the solvent was necessary. Use of DMF did not afford the pure complex as filtration of the product through 0.45 μm filter did not remove the excess unreacted bis-complex, instead, a small amount of black material was seen on the filter which is assumed to be unreacted RuCl$_3$ from earlier steps. DMF was not only solubilising the compound, but impurities and unreacted starting material. Changing the solvent to EtOH did not initially solve the problem of impurities passing through during filtration. $^1$H NMR of the filtrate saw complete disappearance of the polyamide signals, which indicated that the product was not EtOH soluble as assumed.**** Cooling of the solution to 4 °C post-reflux saw the precipitation of the red product that was collected and air-dried.

ESI-MS of LLSP4-Ru did not show the expected parent ions at either 1433.45 m/z (M-H) or 717.23 m/z [M+H] (see Figure A1.26). The spectra also differed significantly from that of LLSP4-(4-CO$_2$H-phen) and did not show the presence of any starting materials (869.3 or 300.5 m/z). In addition to the $^1$H NMR data obtained (discussed further on), this provides some evidence that the compound was made successfully. Therefore the peaks that are

**** The polyamide LLSP4-(4-CO$_2$H-phen) was EtOH soluble prior to reacting with [Ru(dpq)$_2$Cl$_2$].
observed in the mass spectrum are likely to be fragmentation products of the parent ion. It is believed that this complex may have low stability and that ionisation of the complex induces fragmentation despite ESI-MS being a “soft” technique. The assorted and possible fragments of interest are listed in Table 3.7. The importance of these fragmentation products is that they show that the ruthenium complex did coordinate to LLSP4-(4-CO₂H-phen) and the product must have been initially present.

Table 3.7 ESI-MS Fragments.

<table>
<thead>
<tr>
<th>Observed Mass on Charge (m/z)</th>
<th>Ion Fragment</th>
<th>Ion Charge</th>
<th>Calculated Mass on Charge (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>631.3</td>
<td><img src="image1.png" alt="Fragment 1" /></td>
<td>2⁺</td>
<td>631.1</td>
</tr>
<tr>
<td>598.2</td>
<td><img src="image2.png" alt="Fragment 2" /></td>
<td>2⁺</td>
<td>598.1</td>
</tr>
<tr>
<td>584.7</td>
<td><img src="image3.png" alt="Fragment 3" /></td>
<td>2⁺</td>
<td>584.6</td>
</tr>
<tr>
<td>436.9</td>
<td><img src="image4.png" alt="Fragment 4" /></td>
<td>2⁺</td>
<td>436.9</td>
</tr>
</tbody>
</table>

Characterisation of LLSP4-Ru by ¹H NMR (Figure 3.7) showed the addition of several new peaks in the aromatic region when compared to the ¹H NMR of the parent compound LLSP4-(4-CO₂H-phen). These new peaks are indicative of [Ru(dpq)₂Cl₂] coordination to the carboxy-phen ligand. The common resonances shared between the two polyamides in addition to the new ones, are listed in Table 3.8.
### Table 3.8  
$^1$H NMR of LLSP4-(4-CO$_2$H-phen) and LLSP4-Ru.

<table>
<thead>
<tr>
<th>Proton Assignment</th>
<th>Compound</th>
<th>LLSP4-(4-CO$_2$H-phen)</th>
<th>LLSP4-Ru</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aromatic Polyamide Resonances</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.87 (bs, 1H)</td>
<td>9.87 (bs, 1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.85 (bs, 1H)</td>
<td>9.86 (bs, 1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.83 (bs, 2H)</td>
<td>9.83 (bs, 2H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.14 (d, 1H, $J$ = 4.4 Hz)</td>
<td>9.14 (d, 1H, $J$ = 4.2 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.10 (dd, 1H, $J$ = 1.7 / 4.3 Hz)</td>
<td>9.10 (d, 1H, $J$ = 2.8 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.84 (t, 1H, $J$ = 5.6 Hz)</td>
<td>8.85 (t, 1H, $J$ = 5.4 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.48 (dd, 1H, $J$ = 1.7 / 8.1 Hz)</td>
<td>8.47 (d, 1H, $J$ = 8.3 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.12 (d, 1H, $J$ = 9.1 Hz)</td>
<td>8.11 (d, 1H, $J$ = 9.6 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.01 (d, 1H, $J$ = 9.2 Hz)</td>
<td>8.01 (d, 1H, $J$ = 9.1 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.95 (t, 1H, $J$ = 5.6 Hz)</td>
<td>7.95 (t, 1H, $J$ = 5.5 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.78 (m, 1H, $J$ = 4.29 / 8.07 Hz)</td>
<td>7.79 (d, 1H, $J$ = 4.2 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.76 (d, 1H, $J$ = 4.4 Hz)</td>
<td>7.76 (d, 1H, $J$ = 4.1 Hz)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.21 (bs, 2H)</td>
<td>7.21 (bs, 2H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.16 (bs, 2H)</td>
<td>7.16 (bs, 2H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.04 (m, 2H, $J$ = 1.7 / 3.7 Hz)</td>
<td>7.03 (bs, 2H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.90 (d, 1H, $J$ = 1.7 Hz)</td>
<td>6.90 (bs, 1H)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.84 (d, 1H, $J$ = 1.8 Hz)</td>
<td>6.84 (bs, 1H)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Dpq Resonances</strong></th>
<th></th>
<th>9.52 (d, 2H, $J$ = 8.3 Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>9.36 (bs, 4H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.45 (bs, 2H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.35 (m, 4H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.21 (d, 2H, $J$ = 5.6 Hz)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.17 (d, 2H, $J$ = 5.0 Hz)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Aliphatic</strong></th>
<th></th>
<th>3.83 (s, 3H)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3.79 (s, 3H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.40 (t, 2H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.35 (t, 2H)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.43 (t, 2H, $J$ = 6.6 Hz)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.46 (t, 2H, $J$ = 7.2 Hz)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.39 (t, 2H, $J$ = 7.5 Hz)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.91 (t, 2H, $J$ = 7.2 Hz)</td>
</tr>
</tbody>
</table>

The $^1$H NMR spectra for LLSP4-Ru shows the presence of thirty seven aromatic protons; sixteen more than the parent compound LLSP4-(4-CO$_2$H-phen). These sixteen additional protons are consistent with the expected signal; two dpq ligands that have structural symmetry should total to sixteen protons. These new protons are divided between six resonances and not the theoretical value of eight. It is known though that the H7 position on dpq usually appears as one resonance due to overlap, and is demonstrated here by the integrals at 9.36 and 8.35 ppm. The four pyrrole methyl groups are seen at 3.83 (9H) and
3.79 (3H), with broadening of the peaks very evident in comparison to the spectra for LLSP4-(4-CO$_2$H-phen).

Figure 3.7  $^1$H NMR spectrum of LLSP4-Ru, 300 MHz, 35 °C, $d_6$-DMSO. The impurities are denoted by the arrows.

Fluorescence spectroscopy was used to probe some of LLSP-Ru’s DNA binding characteristics. This investigation served two purposes; to provide evidence the ruthenium complex is capable of intercalation whilst coordinated to the polyamide (increased fluorescence in the presence of DNA), and that the complex did successfully coordinate to the polyamide (the polyamide alone does not fluoresce). The fluorescence emission of LLSP4-Ru (0.177 µM) was measured in buffer (10 mM phosphate) alone and then in the presence of ct-DNA (8.33 µM) (Figure 3.8). When the complex interacts with DNA, a large increase in fluorescence emission is seen (44.3 a.u.). This observation confirms that the ruthenium complex was successfully coordinated and that the complex was likely to bind via intercalation. It is known that many intercalative ruthenium octahedral complexes show
increased fluorescence when bound to DNA.\textsuperscript{39, 42, 187} It is hypothesised that when the intercalator binds to DNA all forms of vibrational modes are restricted so excited molecules must release their energy via fluorescence. To determine beyond doubt that this complex is intercalating, in addition to groove binding, further experiments need to be conducted such as viscosity experiments, DNA melting experiments and x-ray diffraction.

\textbf{Figure 3.8}  Fluorescence spectrum of LLSP4-Ru (0.177 µM) and LLSP4-Ru (0.177 µM) + ct-DNA (8.33 µM).

\textbf{3.6 Conclusion}

This chapter reports the synthesis of a novel ruthenium polyamide conjugate LLSP4-Ru. The ancillary ligand dpq was successfully synthesised from the readily available phen. Oxidation of phen produced phendo which underwent condensation with 1,2-diaminoethane to give the dpq product which was characterised by $^1$H NMR. Condensation of crotonaldehyde and 8-aminoquinoline gave the intermediate ligand 4-Me-phen low yields. Sequential oxidation of 4-Me-phen with selenium dioxide and HNO$_3$ yielded 4-CO$_2$H-phen; the linking ligand used to bridge the ruthenium complex and polyamide.

Synthesis of the \textit{bis}-complex [Ru(dpq)$_2$Cl$_2$] was attempted using two different literature methods. The procedure of Anderson \textit{et al.}\textsuperscript{182} consisted of heating at reflux RuCl$_3$.3H$_2$O with dpq and precipitation of the product with acetone. The method of Hua \textit{et al.}\textsuperscript{183} converted
RuCl₃ to an aqua species which was then refluxed in dpq and reduced with SnCl₂ to a Ru (II) species. The preferred method was that of Hua as it produced compound with few impurities in higher yields.

The precursor polyamide LLSP4-(4-CO₂H-phen) was made by solid phase techniques using Fmoc-β-Ala-chlorotrityl resin. Addition of 4-CO₂H-phen to the resin bound polyamide was achieved through coupling reactions using PyBOP coupling agent. A vast excess of 4-CO₂H-phen and coupling agent was required as the coupling was reportedly low yielding. Synthesis of LLSP4-(4-CO₂H-phen) was confirmed by NMR and ESI-MS techniques. NOESY spectra of LLSP4-(4-CO₂H-phen) and LLSP4-Ru could not be used to identify and characterise individual protons due to the resolution acquired and the overlap of many of the peaks.

Heating LLSP4-(4-CO₂H-phen) with [Ru(dpq)₂Cl₂] yielded the target compound LLSP4-Ru which was investigated by several techniques. ¹H NMR showed the successful addition of the metal complex to the polyamide precursor. ESI-MS results did not find the parent ion but fragments were found indicative of successful coordination of the metal complex to the polyamide. Degradation of the compound was evident during the ionisation process and therefore could not be successfully analysed.

Fluorescence spectroscopy showed the compound has basal levels of luminescence that increases significantly upon addition of ct-DNA. It is hypothesised that the ruthenium polyamide conjugate is capable of both binding to the minor groove (akin to Dst) and intercalation via a dpq ligand of the complex. The increase in fluorescence emission upon the binding of LLSP4-Ru to DNA is indicative of an intercalative mode of binding, although it is not definitive evidence.
CHAPTER 4: DNA BINDING

4.1 Introduction

4.1.1 Measuring DNA Binding

The DNA binding constant, $K_b$, is the most commonly accepted measure of DNA binding affinity. The measurement of binding constants has been done using various instruments and the choice of spectrometer is usually determined by the properties of the compound used. Quantitative measurements of a signal are made after changes in DNA concentration or that of the DNA binding molecule.

NMR spectroscopy is used to determine DNA binding constants by measuring chemical shift changes of specific resonances and can be applied to almost any DNA binding compound. This technique however is not efficient in processing a large number of data points and screening of several compounds which requires measurements with specific oligonucleotides.

$K_b$ can also be measured by ultraviolet-visible (UV-Vis) spectroscopy, although the sensitivity of detection of this technique is not as effective as the other methods described here. The low levels of sensitivity require a significantly higher concentration of both DNA and compound in addition to larger titrations.

The use of fluorescence spectroscopy in measuring DNA/drug interactions is highly sensitive, being able to detect small changes in emission. This enables comparatively smaller concentrations of drug and DNA to be used than any other technique available. This method, however, requires a fluorescent compound to work effectively. Without this requirement, a fluorescent intercalator displacement (FID) experiment can be used. In this experiment the DNA sample is saturated with a known fluorescent DNA intercalator so that all binding sites are occupied. The non-fluorescent intercalator is then titrated into the DNA solution and begins to displace bound intercalator. What is measured is a decrease in emission as the bound fluorescent drug is displaced by the non-fluorescent intercalator. Several assumptions
are made using this technique. The compound being measured must have a stronger affinity for DNA than the known intercalator and the energy required to displace the bound molecule is assumed to be the same as the energy required for the drug binding to a free site on DNA. The interactions between the known intercalator and drug are also not taken into consideration making the method flawed.

CD and LD are spectroscopic techniques that can be used to measure the $K_b$ of non-fluorescent compounds and can only be used if one of the molecules in the study is chiral\textsuperscript{††††} such as DNA. CD measures the difference in absorption of left and right circularly polarised light, while LD is the difference in absorption of linearly polarised light parallel and perpendicular to an orientation axis. Regarding these two techniques, CD is the most commonly used to measure DNA binding. There are several advantages of using CD when measuring biological macromolecules such as DNA over other spectroscopic techniques. The measurements can be done in buffers that match biological conditions (such as phosphate buffers) and are not limited to deuterated solvents like NMR. Additionally, it requires much lower concentrations (as with fluorescence spectroscopy) than NMR and UV-Vis spectroscopy.

\subsection{Determination of the DNA Binding Constant, $K_b$}

The determination of binding constants has been best described by Roger and Nordén and is presented here from the modified methods thereof.\textsuperscript{188} The binding of metal complex to DNA can be described by the simple equilibrium:

\[
\text{Free metal complex (M}_f\text{) + DNA binding site (DNA}_f\text{) } \rightleftharpoons \text{Bound metal complex (M}_b\text{)}
\]

The binding constant can therefore be represented by the following equation, where $K_b$ is the apparent binding constant, $M_b$ is the concentration of metal complex bound to DNA and $M_f$ is the concentration of free metal complex.

\textsuperscript{††††} Chiral molecules are not superposable on their mirror images.
\[ K_b = \frac{M_b}{M_f \cdot DNA_f} \]  

(Eq.1)

In order to calculate \( K_b \), the data for \( M_b \) and \( M_f \) must be determined experimentally. Obtaining the spectroscopic data requires the titration of increasing metal complex concentration into a solution of DNA (although the reverse is also possible). Experimental analysis begins with determination of the constant \( \alpha \) using the equation:

\[ M_b = \alpha \rho \]  

(Eq.2)

Where: \( \alpha \) is a constant over the range of binding ratios and \( \rho \) is the observed signal after each titration. The constant \( \alpha \) can be acquired from low binding ratio limits, where all metal complex is assumed bound \( M_f = 0 \).

Thus if total ligand is:

\[ M_t = M_f + M_b \]  

(Eq.3)

Then:

\[ M_t = M_b \]  

(Eq.4)

Rearrangement of Eq.2 for \( \alpha \) and substituting \( M_b \) for \( M_t \) gives:

\[ K_b = \frac{M_b}{M_f \cdot DNA_f} \]  

(Eq.5)

This method to determine \( \alpha \) assumes a uniform binding regime where \( M_f-M_f \) interactions do not affect the signal \( \rho \). Resultantly, non-linear signals detected from \( M_f-M_f \) coupling will be unaccounted for. A method that utilises all data points instead of simply using \( \rho \) when \( M_f = 0 \)
can be used to account for this. The intrinsic method is used when data cannot be obtained from either high or low binding ratios. Eq.1 is then rewritten as:

\[
K_b = \frac{\alpha \rho}{(\text{DNA}_t - \alpha \rho)(M_t - \alpha \rho)} \quad (\text{Eq.6})
\]

Rearranging for \(M_t\) gives:

\[
M_t = \frac{M_t \times \text{DNA}}{\alpha \rho} - \text{DNA}_t + \alpha \rho - \frac{1}{K_b} \quad (\text{Eq.7})
\]

For two different total binding site concentrations, \(\text{DNA}_m^t\) and \(\text{DNA}_n^t\), with the same metal complex concentration where \(M_m^t = M_n^t\), then:

\[
\frac{\text{DNA}_m^t - \text{DNA}_n^t}{\rho^m - \rho^n} = M_t \frac{\rho^m - \rho^n}{\rho^m - \rho^n} + n\alpha
\quad (\text{Eq.8})
\]

Where a plot of:

\[
y = \frac{\text{DNA}_m^t - \text{DNA}_n^t}{\rho^m - \rho^n} \quad (\text{Eq.9}) \quad \text{versus} \quad x = \frac{\rho^m - \rho^n}{\rho^m - \rho^n}
\quad (\text{Eq.10})
\]

has a slope of \(M_t/\alpha\) and a y-intercept of \(n\alpha\), where \(n\) is the binding site size.

The determination of \(\alpha\) from the Intrinsic method allows the calculation of the data required for Scatchard analysis. A Scatchard plot is formed by plotting \(r/M_f\) versus \(r\), where \(r = \)
Scatchard analysis assumes all sites are identical with equal affinities. The linearity implied by these assumptions fails in the presence of multiple binding sites where deviations from linearity are common. It is common for several binding sites to exist in addition to overlaps of these binding sites. Nonlinear plots thus require analysis via nonlinear regression such as the McGee von Hippel model.\textsuperscript{189}

### 4.1.3 Guanosine Binding

The anticancer properties of \textit{cis}-DDP and subsequent platinum based drugs have been linked to the formation of DNA adducts.\textsuperscript{190} The majority of these adducts are seen with the N7 of both guanine and adenine bases. \textit{cis}-DDP coordination adducts form preferentially with guanine bases, accounting for approximately 65\% of total adduction (1,2-d(GpG)).\textsuperscript{47} This information has lead to the investigation of cross-linking ability of contemporary platinum complexes. The \textit{novel} platinum drug BBR3464 has been observed to form 1,4-intrastrand cross-link adducts with DNA.\textsuperscript{93} These adducts form at the N7 positions of guanine residues in the major groove and were characterised by several techniques including ESI-MS, UV-Vis and NMR.

Investigation of cross-link formation in platinum polyamide conjugates has also been researched. Studies by the group of Brabec have examined the formation of coordinate covalent bonds to DNA for the \textit{cis}-DDP-distamycin conjugate Pt-DIST (Figure 4.1).\textsuperscript{162, 163} The results showed cross-links are formed in the minor groove with a significantly higher efficiency than that of \textit{cis}-DDP. It was observed by electrophoretic experiments the targeted bases were between complementary guanosine and cytosine residues.
Coordinate covalent binding of platinum complexes has also been investigated with NMR techniques by monitoring the rate of guanosine adduct formation. Wheate et al. were able to examine the formation of adducts to guanosine with the dinuclear platinum complex \( \text{trans-}[\{\text{Pt}(\text{NH}_3)_2\text{Cl}\}_2\mu-\text{dpzm}]^{2+} \) (di-Pt) (Figure 4.2).\(^{191}\) \(^1\)H NMR analysis of di-Pt incubation (60 °C) with guanosine demonstrated chemical shifts consistent with adduct formation; the disappearance of one resonance saw the appearance of another further downfield. It was also demonstrated that the bonds formed through the N7 position via \(^{195}\)Pt NMR and transcription assays.

Figure 4.1 Structure of the platinum polyamide conjugate Pt-DIST.

Figure 4.2 Structure of the metal complex di-Pt.
4.2 Reagents

Sodium dihydrogen orthophosphate (NaH$_2$PO$_4$) and EDTA was obtained from Ajax Chemicals. Guanosine 5'-monophosphate disodium salt hydrate was purchased from Sigma-Aldrich. The NMR solvents $d_7$-DMF and D$_2$O were purchased from Cambridge Isotope Laboratories. Ct-DNA (sonicated to 2000 bp) was purchased from Gilbco BRL. Disodium hydrogen phosphate 12-hydrate (Na$_2$HPO$_4$) and NaCl was obtained from Merck. Ultra pure water was acquired from a Sartoruis filtration system.

4.3 Instrumentation

UV-Vis spectroscopy was performed on a Cary 300 Bio UV-Vis Spectrophotometer (Varian) and analysed using Cary WinUV® Scan Application Version 3.00.

CD spectra were obtained using a J-810 CD spectrophotometer (JASCO Corporation) with 100 mdeg sensitivity and auto HT voltage settings using a type 32, 10 mm pathlength, CD cell. The spectra were recorded using a scan width of 350 to 200 nm with a continuous scan rate of 500 nm/min and 5 accumulations. The data was analysed using Spectra Analysis® Version 1.51 (Build 02) (JASCO Corporation).

NMR spectra were performed on a 300 MHz Varian Mercury spectrometer equipped with a variable temperature pulsed field gradient probe and analysed using the Windows® based software MestReC (Version 4.5.6). Guanosine experiments were performed as an array where a 256 transient $^1$H NMR spectra was obtained hourly over the course of 64 h at 37 °C.
4.4 Experimental

4.4.1 CD DNA Binding

4.4.1.1 Phosphate Buffer (200 mM NaCl)

Phosphate buffer was prepared by dissolving NaH$_2$PO$_4$.2H$_2$O (0.39 g, 2.50 mmol), Na$_2$HPO$_4$ (0.89 g, 2.50 mmol), EDTA (0.19 g, 0.50 mmol), and NaCl (5.84 g, 0.10 mol) in ~450 mL of ultra pure water. The solution was then adjusted to pH 7 with HCl and made to volume (500 mL). The buffer was then stored at 4 °C.

4.4.1.2 DNA Binding Titrations

Ct-DNA stock solution was prepared by dissolving 24.4 mg of lyophilised ct-DNA in 200 mM NaCl phosphate buffer. An aliquot of 250 μL of DNA stock was then added to 2690 μL of phosphate buffer in the CD cell to give an initial DNA concentration of 2.19 × 10$^{-5}$ M. A stock solution of LLSP4-Pt (1.53 × 10$^{-3}$ M) was prepared in phosphate buffer and titrated into the DNA solution with CD measurements made after each addition. The volumes of the additions used for each titration are provided in the appendix.

4.4.2 Guanosine Binding

Stock solutions of LLSP4-Pt (2.61 mg, 2 mM) and Guanosine (0.81 mg) were prepared in a mix of 300 μL $^d_7$-DMF and 700 μL D$_2$O. The reaction was initiated by mixing 350 μL of each solution in an NMR tube and monitored by $^1$H NMR. Scans were conducted at zero time and then on an hourly basis at 37 °C over 64 h. The free induction decays (FIDs) obtained were then transferred and analysed on MestReC (Version 4.5.6).
CHAPTER 4: DNA BINDING

4.5 Discussion

4.5.1 Binding Constant Determination via CD

Circular dichroism was used to analyse the DNA binding affinity for the platinum polyamide conjugate LLSP4-Pt. The experiment was designed to measure the change in CD of ct-DNA with increasing metal complex concentration. Although chiral, the CD signal is the measurement of the racemic complex and was therefore measured at each point and the baseline subtracted to produce an induced CD spectra.

The titration of ct-DNA (2.19 × 10^{-5} M) with LLSP4-Pt began with small increments (1 μL) from a stock solution of 1.53 × 10^{-3} M. The resultant range of metal complex to DNA ratios measured were from 0.02:1 to 1.5:1. The titrations were done in triplicate, with the spectra for experiment 1 shown in Figure 4.3. The CD maxima at 280 nm for each data point (in each experiment) was then analysed using GraphPad Instat® Version 3.6 to produce the mean and standard deviation at each point. An induced binding curve was then created from this data by plotting the corrected molar ellipticity (Δ[θ])‡‡‡‡, versus LLSP4-Pt concentration (Figure 4.4).

The spectra of the titration (Figure 4.3) shows that upon addition of metal complex there is an increase in the maxima at 280 nm, indicative of a groove binding complex (a decrease in ellipticity is characteristic of DNA intercalators). 188 The induced ct-DNA binding plot begins to plateau off at approximately 1.0 × 10^{-6} M but does not achieve linearity as expected with saturation of a binding site; a change in ellipticity is still occurring with changes in metal complex concentration. It is possible these changes are occurring form a weak secondary mode of binding such as stacking along the exterior of the double helix. This effect has been observed for several platinum/polyamide conjugates in the past. 164

‡‡‡‡ Corrected molar ellipticity was made using the following equation: Δ[θ] = θ/10×C×l (where C is the molar concentration (mole L^{-1}) and l is the cell path length in cm.
Figure 4.3  CD DNA titration #1 (LLSP4-Pt); titration of ct-DNA ($2.19 \times 10^{-5}$ M) with increasing concentrations of LLSP4-Pt in phosphate buffer (200 mM NaCl, 5 mM NaH$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 1mM EDTA). The arrow indicates the direction of change in the spectral features with increasing metal complex concentration.

Figure 4.4  Induced ct-DNA binding plot. The data points (280 nm) are shown with standard deviation bars which were taken and analysed from three sets of data.
The data obtained from the induced ct-DNA binding plot is then processed using Equations 9 and 10 to obtain the Y and X values respectively for the Intrinsic plot shown in Figure 4.5. The slope of this linear regression plot is equal to $M_t/\alpha$ while the y-intercept equals $n\alpha$. The value for $\alpha$ was determined to be $5.54 \times 10^{-1}$ and the binding site size $n$ was 3.95. The $R^2$ value for the slope was calculated to be 0.98 which demonstrates the slope was fitted with minimal error. The value obtained for $n$ at this stage ($n$ can be calculated again with Scatchard analysis) reveals the metal complex is binding to a four base pair site. This data suggests the polyamide component which targets a four base pair sequence is indeed groove binding, while the platinum complex is not involved directly in the binding (coordinate covalent), aside from increased positive charge, as predicted with a high salt concentration (200 mM).

![Intrinsic Plot](image)

**Figure 4.5** Intrinsic Plot. The slope is equal to $M_t/\alpha$ while the y-intercept equals $n\alpha$. The calculated values of $n$ and $\alpha$ are shown in the inset.

The value for $\alpha$ was substituted into Equation 2 where the value for $M_b$ is calculated for each data point by multiplication with the corresponding CD signal $\rho$. The values for $r$ was then calculated by substitution of $M_b$ into the equation $r = M_b/[\text{DNA}]$. The Scatchard plot was made by plotting $r/M_f$ versus $r$ (Figure 4.6).
The binding constant $K_b$ was derived from the slope of the Scatchard plot (slope $= -K_b$) and found to equal $4.1 \times 10^5$ M$^{-1}$. This value is lower in comparison to those reported in the literature for Dst binding to ct-DNA; $0.1 \times 10^6$ and $1.0 \times 10^6$ M$^{-1}$. The salt concentration used for Dst in the study by Dattagupta et al. was 1 M, an extremely high value that was shown to reduce the apparent binding constant compared to a study done at 66 mM salt concentration ($K_b = 0.3 \times 10^6$ and $4.8 \times 10^6$ M$^{-1}$ respectively). The $K_b$ value for LLSP4-Pt is seen to be lower than that reported for Dst (a three ring polyamide) despite the presence of two platinum groups increasing the cationic charge. It is possible that the metal complex may be hindering the binding to some degree and not allowing the polyamide segment to bind effectively in the minor groove.

The binding site size for LLSP4-Pt, $n$, was calculated using the condition where the y-intercept equals $-K_b/n$ and found to be 4.04. This value was in agreement with that obtained via the intrinsic analysis; the metal complex is binding to a site four base pairs in length.

**Figure 4.6** Scatchard Plot. The slope of this plot is equal to $-K_b$ while the intercept value equals $-K_b/n$. The calculated values of $n$ and $K_b$ are shown in the inset.
Guanosine Binding

It has been well established that cisplatin, and derivatives thereof, coordinate preferentially to the N7 of both guanine and adenine bases. Examining the formation of coordinate covalent bonds to guanosine (Figure 4.7) has previously been used to investigate the ability of Pt complexes to form adducts with DNA. This section reports on the binding of the dinuclear platinum complex LLSP4-Pt to the N7 of guanosine, which was monitored by $^1$H NMR over 60 h at 37 °C (Figure 4.8).

![Structure of guanosine]

Figure 4.7  Structure of the nucleoside guanosine, showing the possible coordination sites and numbering system used.

At zero time, the H1' and H8 protons of guanosine are observed at 5.92 and 8.16 ppm. After 1 h the intensity of the H1' and H8 resonances decreased by 4.7 and 7.7%, while three new resonances appeared at 5.98, 8.78 and 8.79 ppm. These results are indicative guanosine N7 platination and are consistent with chemical shifts seen previously. As the reaction proceeds the intensities of the unbound guanosine signals continue to decrease until the 10 h, where the H1' and H8 signals have almost completely disappeared. The appearance of two H8 singlets at 8.78 and 8.79 ppm (designated H8″ and H8′) is attributed to the two possible coordination sites of the complex, which have slightly different chemical environments. The H1' proton remains as a singlet, however as it is not close enough to be significantly affected by the proximal environments of the two platinum centres.
Figure 4.8  Guanosine platination monitored by $^1$H NMR, 300 MHz, 37 °C, 70% D$_2$O / 30% $d_7$-DMF. The H8 proton (unplinated guanosine) can be seen at zero time and visibly disappears by 10 h. Two new resonances appear in its place designated H8' and H8'' (platinated guanosine).

Figure 4.9  Rate of guanosine platination with LLSP4-Pt measured over 60 h and monitored by $^1$H NMR, 37 °C. Approximately 50% saturation is achieved in under 4 h and by 10 h 24% guanosine remains unbound.
The data obtained from the NMR spectra does indicate platination is occurring rapidly, almost reaching coordinative saturation within 15 h (90%). The percentage bound was determined by following integrals of the H8 proton at 8.16 ppm (decreasing integral) and was plotted as a function of time (Figure 4.9).

The reaction rate (Figure 4.9) shows 50% of guanosine is platinated in approximately 4 h and by 10 h 24% remains unbound. The reaction then proceeds very slowly between 11-60 h, never reaching complete coordination within this time.

The reaction was conducted in a 1:1 metal complex to guanosine ratio although it was initially done as a 0.5:1 ratio as this meant there was one platinum group per guanosine residue. The 0.5:1 reaction however did not achieve completion and 60% guanosine remained at unbound even after 60 h at 37 °C. It is believed the binding of a second guanosine residue to the metal complex occurs very slowly. It is hypothesised the binding of the first guanosine residue greatly reduces the affinity of the second from binding, possibly due to steric effects. The binding rate (Figure 4.8) does give evidence to the two distinct binding events. The first binding event can be seen between 1-10 h while the second, much slower, binding event is between 11-60 h.

4.6 Conclusion

This chapter reports upon of the DNA binding characteristics of the novel platinum/polyamide conjugate LLSP4-Pt. Circular dichroism was used to probe the complexes binding affinity for ct-DNA. LLSP4-Pt was titrated into a solution of ct-DNA and measured to a maximum complex to DNA ratio of 1.5:1. The maxima at 280 nm for the data obtained was used to construct a binding plot showing the standard deviation of the three sets of experiments conducted. Complete coordination was not achieved, possibly due to factors such as stacking of the complex, although the phenomena seems to also occur with other polyamides and is not limited to the one investigated here. The CD data obtained indicated
that the compound is groove binding, with an increase in the maxima at 280 nm per addition of metal complex.

The data from the binding plot was then analysed by the Roger and Nordén method to produce the Intrinsic plot from which the value for $\alpha$ was determined ($5.54 \times 10^{-1}$). Linear regression of the data for the Intrinsic plot showed a high accordance with an $R^2$ value 0.98. The value for $\alpha$ enabled the determination of the amount of metal complex bound at each concentration, and thus construction of a scatchard plot. The slope of the scatchard was used to calculate $K_b$ and found to be $4.1 \times 10^5 \text{M}^{-1}$, while the y-intercept provided the binding site size $n$ which equaled 4. The binding constant was lower than that reported for the analogous Dst with the presence of two platinum groups hypothesised to be hindering the groove binding due to steric interactions. The binding site size also provided evidence that the platinum groups were not involved in the binding within the minor groove as expected with the high salt concentration; the 4 bases occupied matched the expected size from the four-ring polyamide component.

Additionally, the ability to form coordinate covalent bonds of LLSP4-Pt with guanosine nucleosides was investigated. The platination of the N7 of guanosine was monitored by $^1$H NMR over 60 h at 37 °C. The H1' and H8 protons of guanosine are observed at 5.92 and 8.16 ppm respectively which shift upon platinum coordination. A new resonance for H1' is seen at 5.98 ppm while two new resonances for H8 are observed at 8.78 and 8.79 ppm as the reaction progressed. The appearance of two resonances for H8 are attributed to the two coordination sites of the complex.

The rate of guanosine platination was also examined showing the progression of the reaction over time with approximately 90% of guanosine platinated within 15 h. The plot of percentage guanosine bound versus time gave evidence there was two binding events as expected with two free platinum groups in the molecule. The first was a rapid binding which was preceded by the slow binding of a second guanosine residue.
CHAPTER 5: CONCLUSIONS

5.1 Results Summary

The cumulative efforts of this thesis was to develop a technique to produce, and investigate the DNA binding potential, of the polyamides LLSP4-Pt and LLSP4-Ru. Synthesis began with developing a solution phase method that could be applied to create any desired polyamide. The idea was to use a monomeric building block that could be used to construct the required polyamide chain length. The precursor molecule 1-methyl-2-pyrrole carboxylic acid was nitrated and then protected via methyl esterification. Hydrogenation of the ester afforded the active monomer that would be used to elongate the chain. Two additions of the monomer to a terminal 1-methyl-2-pyrrole carboxylic acid yielded a polyamide three-ring units long (PyPyPy-COOH). Attempts to elongate the chain passed three ring units were unsuccessful. Several factors were believed to contribute to the inability to progress further. The successively low yields resulted in small amounts of compound available to react with in addition to purification issues. These problems associated with the development of polyamides in solution phase chemistry demanded a more efficient and synthetically viable method.

The synthesis of polyamides using solid phase techniques has been reported in literature and is found to produce compounds with greater ease than those made in solution phase. The coupling reactions undertaken in solution required the removal of the coupling agents and impurities via precipitation and column chromatography. Solid phase removes the necessity of lengthy purification as the product is resin bound allowing impurities to be washed away between each coupling reaction. The advantages of this technique made it a viable alternative to the solution methods.

Solid phase chemistry requires the use of a synthon that enables polyamide chain development while not being susceptible to polymerisation or side reactions. The Fmoc protected pyrrole monomer Py5 was synthesised from adaptations of prescribed literature...
The synthon was made from the relatively inexpensive 1-methylpyrrole as compared to the costly solution phase starting material 1-methyl-2-pyrrole carboxylic acid which added support for development of an alternative method for polyamide synthesis. The acetylation of 1-methylpyrrole yielded Py1 which was then nitrated to yield Py2. tert-Butyl acetylation of produced Py3 which was then hydrogenated yielding the free amine and then Fmoc protected (Py4). Deprotection of the acid (removal of the tert-butyl group) gave the free acid and completed synthon Py5. The high yielding reactions made larger scaled preparations of Py5 possible unlike the experiments for solution phase chemistry which could not be scaled successfully. The coupling reactions for solid phase chemistry required significant amounts of Py5, approximately 1.5 equivalents per addition (1.0-2.5 g of monomer per polyamide made).

Solid phase synthesis began with the production of the precursor polyamide LLSP4-DPA using Fmoc-β-Ala-OH-WANG resin. LLSP4-DPA was designed as a proof of concept molecule, to confirm the solid phase methods worked for the coupling of pyrrole-rings. Initial coupling times used where short (1 h), however, increasing the length of the coupling cycle did not yield more product upon cleavage. The cleaving agent dimethylaminopropyl amine was used to free the polyamide from the solid phase support via aminolysis. Reducing the volume of the cleavage solution under reduced pressure failed to remove the excess solvent and the compound remained in an oily residue. The extension of the aliphatic chain of LLSP4-DPA by addition of dimethylaminopropyl amine gave the polyamide greater non-polar characteristics making it extremely difficult to remove from the amine solution. Precipitation with water aided the recovery of the polyamide but amine impurities were still present and visible in NMR and ESI-MS spectra. These impurities, together with low yielding reactions, necessitated the use of a more appropriate resin for solid phase.

Chlorotrityl resin is an acid labile support that was chosen to test the coupling conditions. LLSP4 was the first polyamide made using the new coupling resin and conditions. This second precursor molecule was made with increased coupling times (~3.5 h) and cleaved
CHAPTER 5: CONCLUSIONS

using TFE and acetic acid. Cleavage produced a free acid at the C-terminus end of the polyamide which enabled precipitation of the compound in water and lyophilisation to produce the dried product. $^1$H NMR and ESI-MS showed synthesis of the polyamide was successful and a vast improvement was seen in terms of purity and yield of the complex. The dinuclear platinum complex LLSP4-Pt was then made using the conditions provided from the synthesis of LLSP4 with some minor modifications. Resin to Py5 coupling equivalents were increased from 1.3 to 2.2 in an effort to maximise yields and decrease potential impurities produced from side reactions and di-Fmoc-Lysine-OH was used as the linker to coordinate the trans-DDP complexes. Following platination of the polyamide the resin was washed several times in brine and water to ensure that the complex formed was a chloride salt and that no excess NaCl remained. Cleavage of the polyamide was afforded with TFE/acetic acid and precipitated by the addition of water which was lyophilised to give the cream coloured compound. Characterisation of of LLSP4-Pt by $^1$H NMR and ESI-MS showed it was successfully synthesised and with only minor impurities present.

The ligands for the novel ruthenium polyamide LLSP4-Ru were made following several literature methods. The ancillary ligand dpq was synthesised via oxidation of phen to phendo, and subsequent condensation with en. Reflux of crotonaldehyde and 8-aminoquinoline gave the intermediate 4-Me-phen which was oxidised to the aldehyde species with selenium dioxide. Subsequent oxidation with nitric acid produced the carboxylic acid 4-CO$_2$H-phen. The synthesis of the complex [Ru(dpq)$_2$Cl] was attempted using two prescribed literature methods. The procedure outlined by Anderson et al.$^{182}$ consisted of simply heating at reflux RuCl$_3$.$3$H$_2$O with dpq, which gave rise to major impurities in the end product. The method of Hua et al.$^{183}$ consisted of conversion of RuCl$_3$.$3$H$_2$O to the aqua species, addition of dpq and reflux of the solution, and the reduction of Ru(III) to Ru(II) using SnCl$_2$. Hua’s was the preferred technique as it produced compound with fewer impurities in higher yields.

The synthesis of the ruthenium polyamide conjugate LLSP4-Ru was made using the the same resin and coupling conditions as for LLSP4-Pt. However, several important distinctions
existed between the syntheses. The addition of 4-CO$_2$H-phen to the resin required use of the coupling agent PyBOP as HBTU fails to activate the acid. A large excess of PyBOP and 4-CO$_2$H-phen is required as the coupling yields are reportedly low yielding; 10.7 equivalents of 4-CO$_2$H-phen was required for coupling, while standard additions of Py5 required only 2.2 equivalents per addition. The polyamide cleaved from the resin was the precursor LLSP4-(4-CO$_2$H-phen) which was reduced in volume, water added and then lyophilised. Heating LLSP4-(4-CO$_2$H-phen) at reflux with [Ru(dpq)$_2$Cl] saw a colour change from a bright yellow to a deep red which signalled the formation of the tris-ruthenium complex LLSP4-Ru. The solution was cooled and a red precipitate formed, which gave further evidence to the insoluble nature of metallo-polyamides seen thus far. $^1$H NMR of both LLSP4-(4-CO$_2$H-phen) and LLSP4-Ru showed the appearance of several peaks corresponding to the dpq and 4-CO$_2$H-phen ligands indicative of successful coordination. NOESY experiments of LLSP4-(4-CO$_2$H-phen) and LLSP4-Ru did not yield any structural information of the polyamides and could not be used to characterise the protons present. ESI-MS results showed the parent ion 869.3 [M+H]$^+$ of LLSP4-(4-CO$_2$H-phen), however the spectra obtained for LLSP4-Ru did not show the expected ions 1433.45 m/z (M-H) or 717.23 m/z [M+H]. The spectra for LLSP4-Ru also failed to show any unreacted starting material. This data alone suggested a change had taken place within the compound and it is most likely that ionisation of the complex is creating severe fragmentation products as shown in the ESI-MS results. Fluorescence spectroscopy of LLSP4-Ru showed the complex did indeed fluoresce where as the precursor LLSP4-(4-CO$_2$H-phen) showed no fluorescence. Upon addition of ct-DNA to the complex a large increase in emission was seen. The increase in fluorescence was believed to be a result of intercalative binding via the dpq ligands.

Despite the successes achieved with the synthetic methods employed for the synthesis of both LLSP4-Pt and LLSP4-Ru, improvement of the technique will be a requirement for future work. A yield of 30.79% and 31.00% for LLSP4-Pt and LLSP4-(4-CO$_2$H-phen) respectively, following cleavage, demonstrates optimisation of the technique being used is necessary. The development of more complex structures, and in larger scales, will require larger quantities of
materials. The synthons required to develop these metallo-polyamides may also require ligands that are more difficult to obtain, such as those that are not commercially available as seen with 4-Me-phen.

Some of the DNA binding characteristics for LLSP4-Pt was explored using CD and NMR techniques. LLSP4-Ru was not investigated as the purity was not suitable for indepth analyses such as binding constant determination. CD was used to probe the DNA binding affinity of LLSP4-Pt to ct-DNA. Metal complex was titrated into a solution of DNA and the maxima at 280 nm was recorded and used to construct a binding plot. Analysis of the plot showed complete saturation was not achieved as there was no definite plateau. This phenomena is seen with other polyamides (unpublished data) and is thought to be a result due to stacking and the nature of dimerisation of the complex. The data from the binding plots was analysed using several equations derived by Rodger et al.\textsuperscript{183} to determine the binding constant $K_b$ and binding site size $n$. The intrinsic plot was used to determine the value for $\alpha$ which is used to calculate the amount of complex bound at any given ratio. Knowledge of the amount of complex bound to DNA at each data point enabled the construction of a Scatchard plot where the gradient is equal to $-K_b$ and the y-intercept $-K_b/n$. The binding constant $K_b$ was found to equal $4.1 \times 10^5$ M$^{-1}$ and the binding site size $n$ equalled 4 base pairs. The binding strength to ct-DNA was comparable, although slightly lower, to analogous polyamides such as Dst. This was unexpected as the presence of the platinum complexes was hypothesised to increase DNA affinity. The DNA binding site size was theoretically expected to be 6 base pairs if the trans-DDP components of the polyamide was involved in the binding. The high salt concentrations though, suppressed the ability for trans-DDP to coordinate or bind to DNA, narrowing the binding portion of the molecule to the pyrrole rings alone; LLSP4-Pt should only bind to 4 bases. This theory agreed with the data obtained from Scatchard analysis.

The ability of LLSP4-Pt to bind irreversibly to DNA was investigated using NMR spectroscopy. The platination of the N7 of guanosine was monitored by $^1$H NMR over 60 h at
37 °C. Upon binding of LLSP4-PT a change in the guanosine resonances were seen. The H1' and H8 proton chemical shifts disappeared over time and new resonances appeared downfield in their stead. Two extra resonances appeared for the H8 proton and these were attributed to the individual binding of the two trans-DDP components of the molecule. The rate of guanosine platination was examined by plotting the percentage of guanosine bound versus time. It was observed that 90% of guanosine is platinated within 15 h and there is some indication that two binding events are occurring; a direct result of two platinum complexes with different chemical environments. The first event was shown to occur rapidly while the second proceeded at a much slower rate.

5.2 Future Work

The synthesis of the complexes LLSP4-Ru and LLSP4-Pt have shown through their yields and purity, that refinements in the synthetic strategies adopted may be needed. The investigation of other resins and modulation of the conditions are alternatives that can be explored whilst using solid phase synthesis. In addition, purification of the end products via HPLC remains another possibility that is reported in the literature. Further analysis of the DNA binding characteristics of these complexes also needs to be explored. The binding constant of LLSP4-Ru could be determined using fluorescence spectroscopy (once the complex is sufficiently purified). DNA Melting and viscosity experiments would also be a valuable analysis, as this would add support to the intercalative ability of LLSP4-Ru. Cell line testing for both complexes could reveal any biological activity, in particular, LLSP-Pt which could display interesting results in cancer cell lines with the ability to form unique DNA adducts.

The investigation of metal complex polyamide conjugates in this work was limited to linear groove binders capable of dimerisation. The next generation of complexes in this line of research would be to synthesise hairpin metallo-polyamides. This would produce complexes that bind to DNA sequences with a higher fidelity than the linear counterparts (although this
was not examined in this thesis). Current research is underway by the Aldrich-Wright group to create the bifunctional synthon trans-diamino(ethane-1,2-diamino)fluoromethoxycarbonyl-2-aminoacetate platinum(II) (DEFMAP) (Figure 5.1). DEFMAP will serve as a linker to make unique hairpin polyamides that also carry the functionality of a metal complex. The inclusion of metal complexes will add positive charges and hopefully aid in solubilising these class polyamides.

![Figure 5.1](image_url) The novel hairpin polyamide linker DEFMAP.

DEFMAP will be coupled to polyamide chains using standard coupling techniques via solid phase through carboxylic acid of the glycine ligand. Deprotection of the Fmoc group will allow subsequent additions of more Py or Im rings and extension of the polyamide. This linker will also make mixed metal polyamides possible with the addition of a ruthenium metal complex at the end of the chain.

The intercalative metal complexes that can be used in these next generation hairpin polyamides could vary greatly in functionality and in binding modes, such as the complex DNHP4-Ru$_2^{§§§}$ shown in Figure 5.2. Addition of a second ruthenium intercalator would improve the polyamides affinity to DNA via increased positive charge of the complex (4+) and the presence of two intercalative groups, which theoretically would “clamp” the molecule in place. The placement of [Ru(dpq)$_2$(η-phen)]$^{2+}$ (where η is the bridging position on the ligand to the polyamide) on the hairpin linker could hinder the complexes ability to

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§§§ Nomenclature: Dinuclear hairpin polyamide, 4 rings in length, 2 ruthenium nuclei.
bind due to steric interactions. It would be prudent to investigate the optimal aliphatic linker length to enable the free range of movement of the complex so intercalation is possible.

Replacing the dpq ligand in these complexes with dppz could provide the hairpin polyamide with the “light switch” effect seen in other dppz complexes. While other fluorescent complexes tend to emit at basal levels while in solution, dppz requires intercalation into DNA to fluoresce. This can provide a more accurate analysis of the localisation of the complex within a cell, and determine if the complex is penetrating the nucleus with minimal interference from the unbound form of the complex.

![Image of a molecule](image_url)

**Figure 5.2** DNHP4-Ru2; part of the next generation hairpin metallo-polyamides.

Platinum and ruthenium mixed metal complexes are also currently under investigation by the Aldrich-Wright group. The complex \([trans-\{PtCl(NH_3)_2\}_2\mu-\{Ru(Me_2bpy)_2(bpyL)\}]^{4+}\) a novel molecule comprised of two trans-DDP groups both link to a central Ru(II) polypyridyl complex (Figure 5.3). Like BBR3464, the complex has an overall charge of 4+, which should result in a high affinity for DNA. The six-carbon chain linkers to the trans-DDP complexes also emulate the structure of BBR3464 and might also be capable of the unique 1,4-interstrand cross-links adducts. The adduct formation of BBR3464 are capable of overcoming cis-DDP resistant tumors and would be highly advantageous for this mixed metal complex. The cellular uptake and localisation of the complex could be observed via fluorescence microscopy. Replacement of the aliphatic linkers with short polyamides also...
provides other opportunities to explore mixed metal polyamide conjugates. Replacement of the ruthenium ancillary ligands to ones capable of intercalation could see the development of molecules capable of three distinct binding modes; groove binding, intercalation, and coordinate covalent bond formation.

![Figure 5.3](image_url)

**Figure 5.3** The complex \[\text{[trans} \cdot \text{PtCl(NH}_3)_2\text{]}_{2\mu} \cdot \text{[Ru(Me}_2\text{bpy)}_2(bpyL)}\]^{4+}.

Investigation into bis intercalating polyamides is currently underway by the Dervan group. The symmetric hairpin polyamide-acridine conjugate \((\text{ImPyPy-} \beta \text{-Do-Acr})_2(\text{CH}_2)_6\) is a bisintcalating polyamide consisting of two bridged linear strands. Studies have shown the complex is capable of binding specific DNA sites at subnanomolar concentrations and unwinding of DNA by more than 30°.

The ability to target specific genes and control their expression via sequence specific molecules holds a vast potential of previously unexplored therapeutics for diseases such as cancer, which remains as one of the most feared and fervently studied diseases today. While the paths of research are exploring many areas of possible cures, the investigation of metallo-polyamides combined with sequence specific technology is yet to be fully utilised.
REFERENCES


96. Arcamone, F.; Bizioli, F.; Canevazzi, G.; Grein, A. Distamycin and Distacin. *German Pat.* 1,027,667, **1958**.


Appendix

A1. NMR Spectra

Figure A1.1: $^1$H NMR of 1-methyl-4-nitropyrrrole-2-carboxylic acid.

Figure A1.2: NOESY of 1-methyl-4-nitropyrrrole-2-carboxylic acid.

Figure A1.3: $^1$H NMR of methyl 1-methyl-4-nitropyrrrole-2-carboxylate.

Figure A1.4: NOESY of methyl 1-methyl-4-nitropyrrrole-2-carboxylate.

Figure A1.5: $^1$H NMR of methyl-1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylate (PyPyCO$_2$CH$_3$).

Figure A1.6: $^1$H NMR of 1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylic acid (PyPyCO$_2$H).

Figure A1.7: NOESY of 1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylic acid (PyPyCO$_2$H).

Figure A1.8: $^1$H NMR of methyl 1-methyl-4-{1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl}-amino)-1H- pyrrole-2-carboxylate (PyPyPyCO$_2$CH$_3$).

Figure A1.9: $^1$H NMR of 1-methyl-4-{1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl}-amino)-1H- pyrrole-2-carboxylic acid (PyPyPyCO$_2$H).

Figure A1.10: NOESY of 1-methyl-4-{1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl}-amino)-1H- pyrrole-2-carboxylic acid (PyPyPyCO$_2$H).

Figure A1.11: $^1$H NMR of 2-trichloroacetyl-1-methylpyrrole (Py1).

Figure A1.12: NOESY of 2-trichloroacetyl-1-methylpyrrole (Py1).

Figure A1.13: $^1$H NMR of 4-nitro-2-trichloroacetyl-1-methylpyrrole (Py2).

Figure A1.14: NOESY of 4-nitro-2-trichloroacetyl-1-methylpyrrole (Py2).

Figure A1.15: $^1$H NMR of tert-butyl-4-nitro-1-methylpyrrole-2-carboxylate (Py3).

Figure A1.16: NOESY of tert-butyl-4-nitro-1-methylpyrrole-2-carboxylate (Py3).
**Figure A1.17:** $^1$H NMR of tert-butyl 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate (Py4).

**Figure A1.18:** NOESY of tert-butyl 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate (Py4).

**Figure A1.19:** $^1$H NMR of 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid (Py5).

**Figure A1.20:** NOESY of 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid (Py5).

**Figure A1.21:** $^1$H NMR of LLSP4-DPA.

**Figure A1.22:** $^1$H NMR of LLSP4.

**Figure A1.23:** $^1$H NMR of LLSP4-Pt.

**Figure A1.24:** $^{195}$Pt NMR of LLSP4-Pt.

**Figure A1.25:** $^1$H NMR of 1,10-phenanthroline-5,6-dione (phendo).

**Figure A1.26:** $^1$H NMR of dipyrido[3,2-d:2'3'-f]quinoxaline (dpq).

**Figure A1.27:** $^1$H NMR of 4-methyl-1,10-phenanthroline (4-Me-phen).

**Figure A1.28:** $^1$H NMR of 4-carboxy-1,10-phenanthroline (4-CO$_2$H-phen).

**Figure A1.29:** $^1$H NMR of LLSP4-(4-CO$_2$H-phen).

**Figure A1.30:** $^1$H NMR of LLSP4-Ru.

**A2. ESI-Mass Spectra**

**Figure A2.1** ESI-MS of LLSP4-DPA.

**Figure A2.2** ESI-MS of LLSP4.

**Figure A2.3** ESI-MS of LLSP4-Pt.

**Figure A2.4** ESI-MS of LLSP4-(4-CO$_2$H-phen).

**Figure A2.5** ESI-MS of LLSP4-Ru.
A3. Circular Dichroism spectra and calculations: ct-DNA titrations

Table A.1  LLSP4-Pt concentration ranges.

Figure A3.1  CD DNA titration #1

Figure A3.2  CD DNA titration #2

Figure A3.3  CD DNA titration #3

Figure A3.4  Induced ct-DNA binding plot.

Figure A3.5  Intrinsic plot of combined results.

Figure A3.6  Scatchard plot of combined results.
A1. NMR Spectra

Figure A1.1: $^1$H NMR of 1-methyl-4-nitropyrrrole-2-carboxylic acid, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.2: NOESY of 1-methyl-4-nitropyrrrole-2-carboxylic acid, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.3: $^1$H NMR of methyl 1-methyl-4-nitropyrrrole-2-carboxylate, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.4: NOESY of methyl 1-methyl-4-nitropyrrrole-2-carboxylate, 300 MHz, 35 °C, $D_{6}$-DMSO.
**Figure A1.5:** $^1$H NMR of methyl-1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylate (PyPyCO$_2$CH$_3$), 300 MHz, 25 °C, CD$_3$OD.
Figure A1.6: $^1$H NMR of 1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylic acid (PyPyCO$_2$H), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.7: NOESY of 1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-pyrrole-2-carboxylic acid (PyPyCO₂H), 300 MHz, 35 °C, d₆-DMSO.
Figure A1.8: $^1$H NMR of methyl 1-methyl-4-([1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl]-amino)-1H- pyrrole-2-carboxylate (PyPyPyCO$_2$CH$_3$), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.9: $^1$H NMR of 1-methyl-4-((1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl]-amino)-1H- pyrrole-2-carboxylic acid (PyPyPyCO$_2$H), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.10: NOESY of 1-methyl-4-([1-methyl-4-[(1-methyl-1H-pyrrole-2-carbonyl)-amino]-1H-pyrrole-2-carbonyl]-amino)-1H- pyrrole-2-carboxylic acid (PyPyPyCO$_2$H), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.11: \(^1\)H NMR of 2-trichloroacetyl-1-methylpyrrole (Py1), 300 MHz, 25 °C, CDCl\(_3\).
Figure A1.12: NOESY of 2-trichloroacetyl-1-methylpyrrole (Py1), 300 MHz, 25 °C, CDCl₃.
Figure A1.13: $^1$H NMR of 4-nitro-2-trichloroacetyl-1-methylpyrrole (Py2), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.14: NOESY of 4-nitro-2-trichloroacetyl-1-methylpyrrole (Py2), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.15: $^1$H NMR of tert-butyl-4-nitro-1-methylpyrrole-2-carboxylate (Py3), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.16: NOESY of tert-butyl-4-nitro-1-methylpyrrole-2-carboxylate (Py3), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.17: $^1$H NMR of tert-butyl 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate (Py4), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.18: NOESY of tert-butyl 4-[9-fluorenlymethoxycarbonyl]amino]-1-methylpyrrole-2-carboxylate (Py4), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.19: $^1$H NMR of 4-[(9-fluorenylmethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid (Py5), 300 MHz, 35 °C, $d_6$-DMSO.
**Figure A1.20:** NOESY of 4-[(9-fluorenlymethoxycarbonyl)amino]-1-methylpyrrole-2-carboxylic acid (Py5), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.21: $^1$H NMR of LLSP4-DPA, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.22: $^1$H NMR of LLSP4, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.23: $^1$H NMR of LLSP4-Pt, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.24: $^{195}$Pt NMR of LLSP4-Pt, 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.25: $^1$H NMR of 1,10-phenanthroline-5,6-dione (phendo), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.26: $^1$H NMR of dipyrido[3,2-d:2'3'-f]quinoxaline (dpq), 300 MHz, 35 °C, CD$_3$CN.
Figure A1.27: $^1$H NMR of 4-methyl-1,10-phenanthroline (4-Me-phen), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.28: $^1$H NMR of 4-carboxy-1,10-phenanthroline (4-CO$_2$H-phen), 300 MHz, 35 °C, $d_6$-DMSO.
Figure A1.29: $^1$H NMR of LLSP4-(4-CO$_2$H-phen), 300 MHz, 35 °C, $D_6$-DMSO.
Figure A1.30: $^1$H NMR of LLSP4-Ru, 300 MHz, 35 °C, $D_6$-DMSO.
A2. ESI-Mass Spectra

Figure A2.1: ESI-MS of LLSP4-DPA.
Figure A2.2: ESI-MS of LLSP4.
Figure A2.3: ESI-MS of LLSP4-Pt.
Figure A2.4: ESI-MS of LLSP4-(4-CO$_2$H-phen).
Figure A2.5: ESI-MS of LLSP4-Ru.
A3. Circular Dichroism spectra and calculations: ct-DNA titrations

CD spectra were obtained by titration of ct-DNA (2.19 × 10^{-5} M, 2940 μL) with increasing concentrations of LLSP4-Pt in phosphate buffer (200 mM NaCl, 5 mM NaH₂PO₄, 5 mM Na₂HPO₄, 1 mM EDTA). The concentration ranges of LLSP4-Pt are listed in Table A1.

Table A.1 LLSP4-Pt concentration ranges.

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</table>
Figure A3.1  CD DNA titration #1 (LLSP4-Pt); titration of ct-DNA (2.19 × 10^{-5} M) with increasing concentrations of LLSP4-Pt in phosphate buffer (200 mM NaCl, 5 mM NaH_{2}PO_{4}, 5 mM Na_{2}HPO_{4}, 1 mM EDTA). The arrow indicates the direction of change in the spectral features with increasing metal complex concentration.

Figure A3.2  CD DNA titration #2 (LLSP4-Pt); titration of ct-DNA (2.19 × 10^{-5} M) with increasing concentrations of LLSP4-Pt in phosphate buffer (200 mM NaCl, 5 mM NaH_{2}PO_{4}, 5 mM Na_{2}HPO_{4}, 1 mM EDTA). The arrow indicates the direction of change in the spectral features with increasing metal complex concentration.
Figure A3.3  
CD DNA titration #3 (LLSP4-Pt); titration of ct-DNA ($2.19 \times 10^{-5}$ M) with increasing concentrations of LLSP4-Pt in phosphate buffer (200 mM NaCl, 5 mM NaH$_2$PO$_4$, 5 mM Na$_2$HPO$_4$, 1 mM EDTA). The arrow indicates the direction of change in the spectral features with increasing metal complex concentration.

Figure A3.4  
Induced ct-DNA binding plot. The data points (280 nm) are shown with standard deviation bars which were taken and analysed from three sets of data.
**Figure A3.5**  Intrinsic plot; the slope is equal to $M/na$ while the y-intercept equals $na$. The calculated values of $n$ and $\alpha$ are shown in the inset.

**Figure A3.6**  Scatchard plot; the slope of this plot is equal to $-K_b$ while the intercept value equals $-K_b/n$. The calculated values of $n$ and $K_b$ are shown in the inset.