

Supramolecular approaches for controlling the interactions between metal complexes and DNA

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Declaration of Authorship

I declare that the work herein is original and was carried out and written by the author, Timothy Kench. All contributions by collaborators are acknowledged and all references to published work are listed in the bibliography.

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Abstract

G-Quadruplexes (G4) are a type of non-canonical DNA structure, formed from guanine-rich single-stranded DNA, which folds into a four-stranded structure of stacked tetrads. These highly stable structures have been shown to form in a range of physiological conditions and interest for G4 DNA as a target for anticancer therapeutics has increased dramatically as evidence for their existence in vivo has mounted. This is due to the identification of G4 forming sequences at the telomeres and in key oncogene promotor regions.

Much work has gone into the development of small molecules which can interact with G4 DNA, both as potential drugs to target disease and as new optical probes with which to continue to study G4 DNA. Our group is interested in the potential for metal complexes to be used as G4 binders; specifically, metal salphens. Pt^{II} -salphens show excellent G4 stabilisation, are phosphorescent and have controllable redox states. This work therefore aimed to utilise Pt^{II} -salphens as a tool for various applications. This has included the development of emissive dimeric ligand systems which can be used to selectively target higher order G4 DNA structures potentially found at the telomeres.

We have also developed several approaches aimed at caging Pt^{II} -salphens for triggered release. The first of these involved the synthesis of a set of interlocked compounds (rotaxanes) with cleavable stopper groups. When enclosed, the Pt^{II} -salphen could not interact with DNA. Upon exposure to either light or an enzyme the rotaxanes broke down, releasing the Pt^{II} -salphen. Both the cytotoxicity and cellular localisation of the rotaxanes could be controlled via this mechanism.

Finally, a series of redox controlled Pt^{IV} -salphens with improved stability were synthesised. These compounds were based on an inactive form of a Pt^{II} -salphen, which is activated by bio-reductants found in higher concentrations in tumour environments.

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Abbreviations

δ	NMR chemical shift
ADOTA	azadioxa-triangulenium
AMT	active-metal template
aq.	aqueous
anh.	anhydrous
cat.	catalytic
CD	circular dichroism
conc.	concentrated
CuAAC	copper(I)-catalysed azide–alkyne cycloaddition
CT	calf thymus
d	doublet
DAOTA	diazaoxa-triangulenium
DCM	dichloromethane
DFT	density functional theory
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionisation
HB	hydrogen-bonding
MS	mass spectrometry
NMR	nuclear magnetic resonance spectroscopy
NMP	N-Methylpyrrolidone
NOESY	nuclear Overhauser Effect Spectroscopy
PEG	polyethylene glycol

ROESY	rotating-frame nuclear Overhauser effect correlation spectroscopy
RT	room temperature
sat.	saturated
T _m	melting temperature
TBA	tetrabutylammonium
TFA	trifluoroacetic acid
TO	thiazole orange
TLC	thin-layer chromatography

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Chapter 1

Introduction

The structure and function of DNA is inherently linked. In humans, each cell nuclei contains approximately 3×10^9 base pairs of DNA, divided into 23 chromosomes, weighing 6-7 picograms and encoding 20,000 genes. This enormous information storage and transfer is achieved through the manipulation of the structure of DNA. Constructed from a sequence of nucleotides, each subunit of DNA contains a nitrogenous base bound to a sugar phosphate moiety. A strand is formed through the covalent attachment of subunits by phosphate bonds; the double helix is constructed through hydrogen bonding between base pairs on opposing strands and it is the reversible nature of hydrogen bonding which bestows DNA with such remarkable properties for the storing and writing of biological data. Whilst the majority of DNA information transfer is achieved through the unwinding of double helical DNA, there are many additional forms and functions of such a highly polymorphic structure.

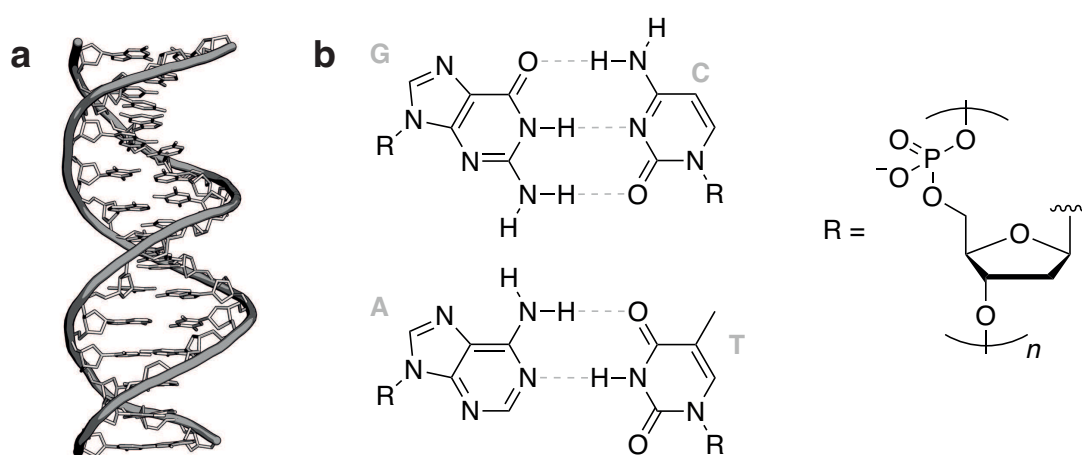


Figure 1.1 The structure of DNA: (a) the double helix (PDB: 1XAV) and (b) the favoured base pairing between GC and AT.

The double helical form of DNA is primarily dictated by complementary hydrogen bonding between specific base pairs; guanine (G) and cytosine (C), and adenine (A) and thymine (T) (Watson-Crick base pairing, Figure 1.1b). In each case a purine (G and A) is bound to a pyrimidine (C and T) with the GC pair containing three hydrogen bonds and the AT pair containing two. Through the repeated stacking of canonical base pairing between strands, a hydrophobic inner channel is formed. The glycosidic bond angle, in conjunction with a driving force to reduce surface hydrophobicity, causes a right handed helical shape with major and minor grooves (B-DNA).

1.1 Non-canonical DNA

DNA, however, is not limited to just the B form and under certain biological conditions a variety of different structures are possible. For example, B-DNA is just one of three biologically relevant helix structures:^[1] A-DNA (Figure 1.2a) has been shown to form under strongly dehydrating conditions and is a more compact form thought to protect DNA under harsh conditions,^[2] whilst Z-DNA (Figure 1.2b) is a left handed helix with a zigzag shape and major and minor grooves of similar width. It has been demonstrated to form under negative supercoiling and high salt conditions.^[3]

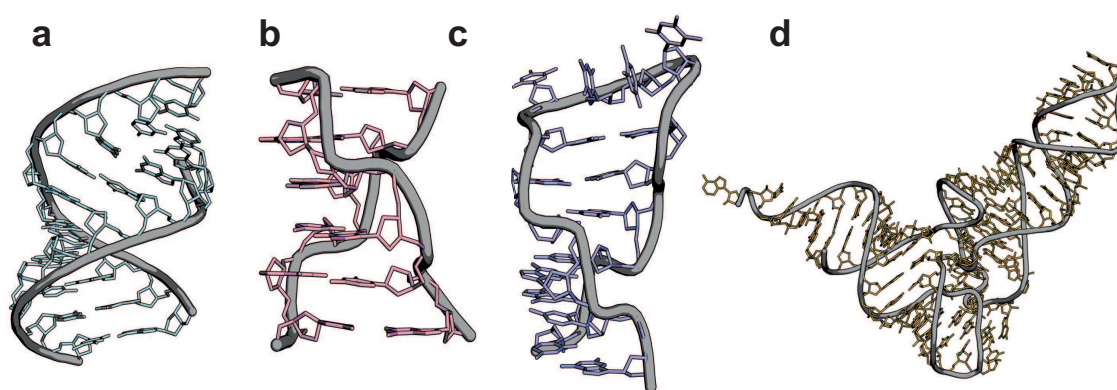


Figure 1.2 Non-canonical DNA structures: (a) an A-DNA helix (PDB: 440D), (b) a Z-DNA DNA helix (PDB: 1V9G), (c) an example of a DNA hairpin (PDB: 1D16) and (d) an DNA cruciform structure (PDB: 1EHZ).

Additionally, in order for information to be accessed from DNA, the helical structure needs to be disrupted and strands separated. During DNA replication, transcription and translation, the presence of single-stranded DNA or RNA increases the number of potential secondary structures that can form. For example, hairpins (Figure 1.2c), in which a single strand of mRNA folds intramolecularly generating base pairing, are thought to play an important role in protein recognition.^[4] Cruciform DNA (Figure 1.2d) can form in DNA sequences containing inverted repeats which can base pair with each other, with a stem loop in each strand creating the cruciform shape.^[5]

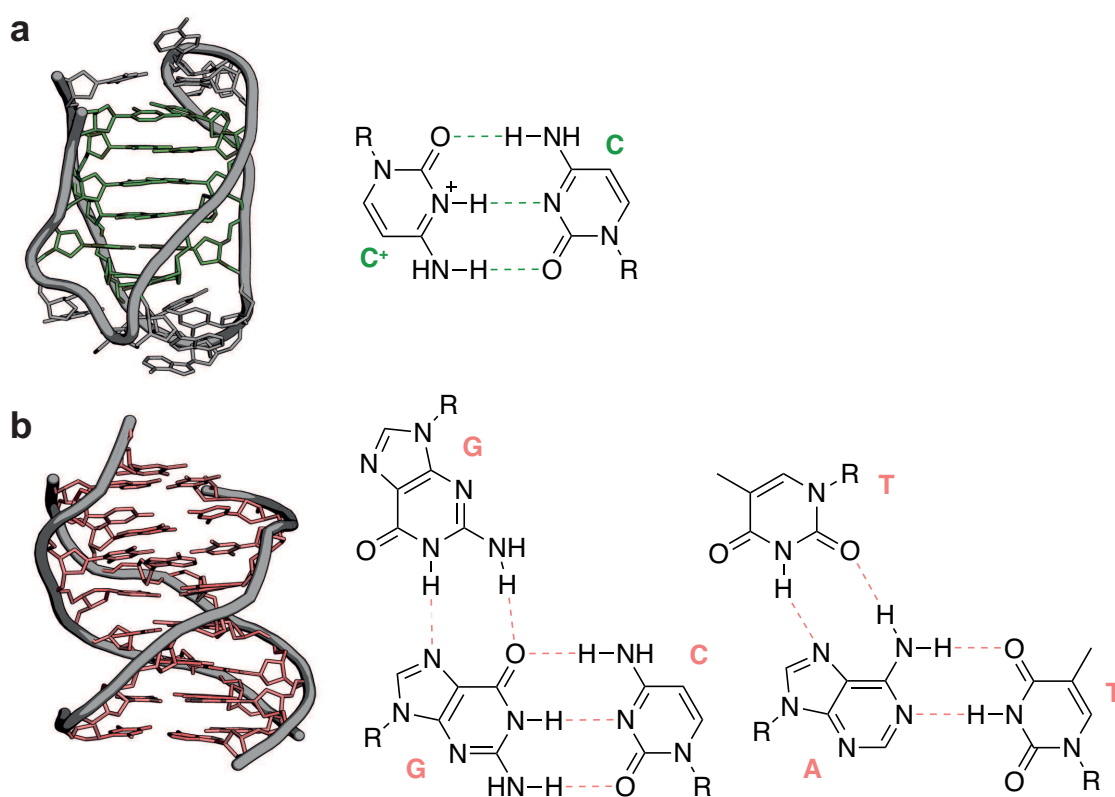


Figure 1.3 Non-canonical DNA base pairing: (a) an i-motif (PDB: 1EL2) with C-C⁺ base pairing and (b) a triplex helix (PDB: 1D3X) with G-C-G and T-A-T base pairing.

Another way in which DNA can form alternative stable structures is through non-canonical base pairing. All the structures previously listed obey Watson-Crick base pairing in which G is always paired with C and A is always paired with T. This, however, does not always have to be the case. In structures known as i-motifs C can pair with C⁺ to form a four stranded structure in which each base pair crosses through the other (Figure 1.3a).^[6,7] Triplex DNA relies on Hoogsteen base pairing, which utilises the N7 of the purine

as a hydrogen bond acceptor and the C6 amino group of the pyrimidine base as a hydrogen bond donor. This drastically changes the angles between bases and means that in the case of triplexes, a third strand can be incorporated (Figure 1.3b).^[8] The ability of non-canonical DNA secondary structures to regulate protein binding and gene expression has led to a great deal of investigation into these structures as druggable targets, with perhaps the greatest emphasis so far on G-quadruplex DNA.^[9,10]

1.2 G-Quadruplexes

G-Quadruplexes (G4s) are formed through Hoogsteen base pairing, in which guanine displays hydrogen bonding interactions with itself to form a tetrad structure (Figure 1.4). Whilst this behaviour of guanine was first posited in 1962,^[11] research on G4s only significantly increased when, in the 1980s, it was demonstrated that telomeric DNA sequences displayed the same sort of behaviour *in vitro*.^[12] Since then, the field has widened considerably, with a focus on the elucidation of their biological function and their potential as a target for various therapeutic strategies.^[13–16]

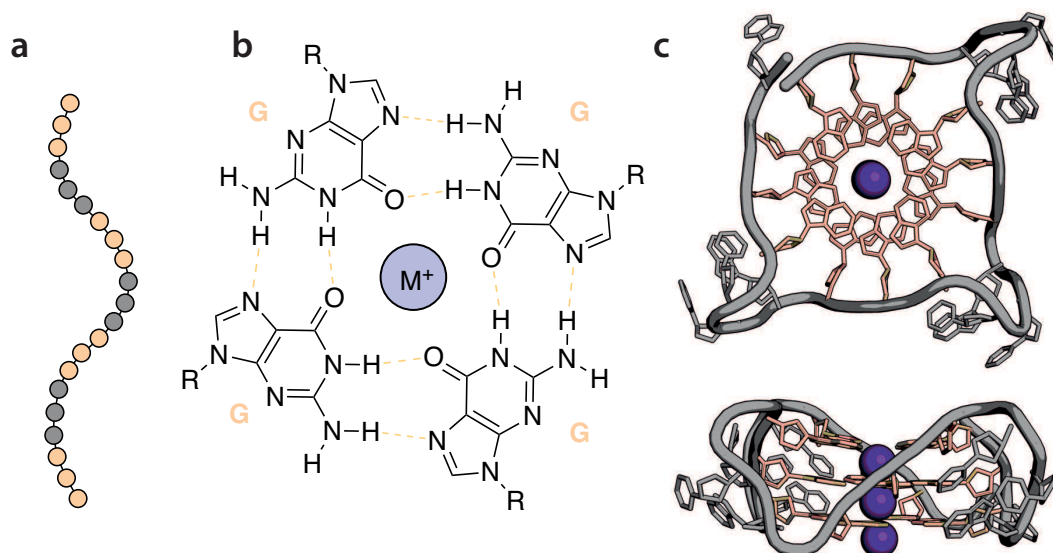


Figure 1.4 G-Quadruplex formation: (a) single stranded DNA with multiple G-runs, (b) G-tetrad formed from four guanines through Hoogsteen base pairing and (c) a crystal structure of a G4 unit (PDB: 1KF1) showing side view of stacked tetrads and loops with K⁺ ions occupying the central ion channel.

The formation of a G-tetrad from four guanines has a number of important structural consequences. Firstly, the large planar surfaces that form can ideally stack on one another to minimise hydrophobicity to the intrinsic aqueous environment. Secondly, the placement of four O6 oxygens all pointing inwards creates a negatively charged central channel which can be occupied by certain cations. G-Quadruplexes can incorporate a number of different cations, with various stabilising effects ($K^+ > Na^+ > NH_4^+ > Li^+$, with Na^+ and K^+ being the most physiologically relevant).^[17,18] The synergistic effect of electrostatic interactions, hydrogen bonding, $\pi - \pi$ stacking and Van der Waals forces leads to a highly stable four-stranded structure.

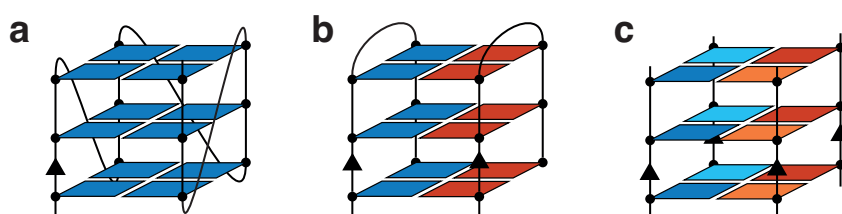


Figure 1.5 G4 polymorphism: G4 DNA structures formed from (a) one, (b) two and (c) four separate strands, with the guanine bases from each strand highlighted in different colours.

Outside of these few essential requirements, G4 structures exhibit a significant degree of polymorphism. They can form from one, two, three or four strands containing single or multiple G-rich tracts to give rise to inter- or intramolecular G-quadruplexes (Figure 1.5). In a biological context intramolecular G4s are of greatest interest as a single strand of DNA with multiple G-rich tracts in close proximity has an entropic driving force to self-fold as opposed to multiple strands coming together. The process of folding is itself highly complex and has been demonstrated to dynamically pass through a number of different structures including G-triplex and G-hairpin structures with relatively little cation dependence.^[19]

A number of basic G4 topologies have been defined, depending on the direction of each strand, the conformation of each nucleotide as either syn or anti and the type of loop connecting each strand (Figure 1.6). Whilst many others are also theoretically possible, the structures shown in Figure 1.6 are all highly biologically conserved and have been characterised by crystallographic methods. Parallel G4s are defined by each strand being aligned

in the same direction; each base is in a syn conformation and a propeller type loop connects each strand. In anti-parallel G4s the strand direction is reversed between edges; if there are three lateral loops the structure is termed a 'chair-type' structure, whereas with a diagonal loop the structure is 'basket-type'. The 'hybrid-type 3+1' and 'hybrid-type 2+2' structures involve a combination of propeller and lateral type loops.

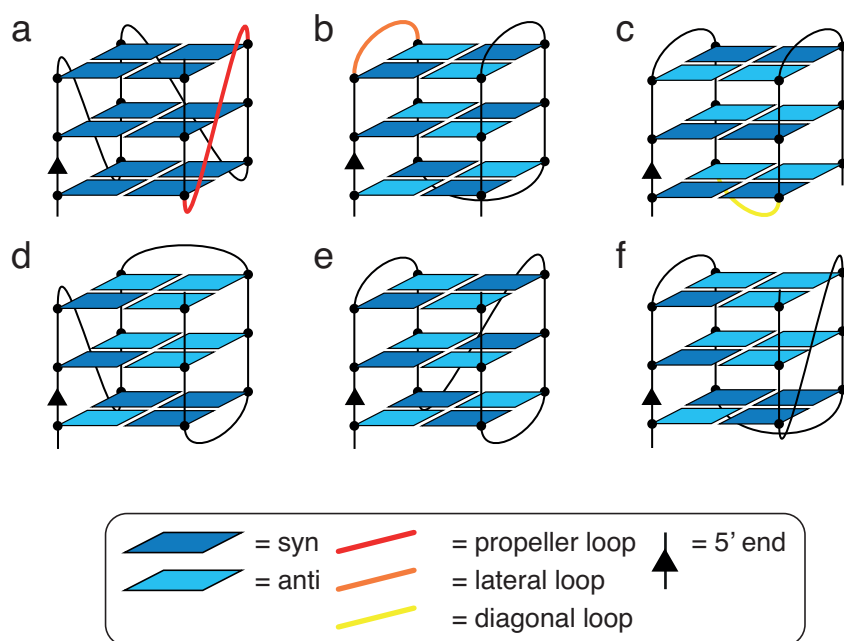


Figure 1.6 G4 polymorphism: (a) parallel topology, (b) chair topology, (c) basket topology, (d) hybrid topology, (e) hybrid (2+2) topology and (f) hybrid (3+1) topology.

Whether a certain topology forms is dependent on the interplay of various parameters. For example, the size of Na^+ means that it sits within the G-tetrad plane, whereas K^+ cations are found between adjacent layers; consequently the K^+ structure is more compact. For G4s formed from the human telomeric (HTelo) sequence, the presence of Na^+ cations leads to an antiparallel structure whereas in K^+ buffers it varies between a parallel form in the crystal structure,^[20] and a mixture of parallel/antiparallel hybrid structure and chair conformations in solution.^[21] In an attempt to more closely recreate a cellular environment,^[22] the effect of molecular crowding agents on G4 topology have been investigated; molecular crowding can induce a conformational switch from antiparallel to parallel of a d(G₄T₄G₄) sequence whereas polycationic crowding does not have the same effect.^[23] Furthermore, molecular crowding conditions (40% w/v PEG) and K^+ buffer induce a switch in HTelo G4

topology from hybrid structure to a parallel stranded structure and show increased stability.^[24]

G4 topology and folding is also highly dependent on loop length;^[25] at the extremes the number of bases in each loop can vary from one (restricting the structure to a parallel topology)^[26] and can be extended up to thirty bases.^[27] Furthermore, the role of flanking bases can dictate which topology folds.^[28] Additional factors can cause a conformational switch; an investigation into the effect of pH and temperature on a sequence of duplex DNA capable of forming a G4 and an i-motif in each strand demonstrated how the equilibrium between the different structures could be shifted.^[29] Recent work has shown how abasic site formation (through the presence of reactive oxygen species)^[30] can have an effect on G4 stability depending on where the abasic site occurs. The ability to still form G4s is generally preserved and in some cases can be stabilised using a fifth G-track.^[31,32] The polymorphism of G4 structures has been extensively reviewed,^[33–37] including a review of some of the more exotic G4 structures which include bulges, long loops and vacant base sites.^[38] Examples of a variety of G4 forming sequences are listed in Table 1.1.

Table 1.1 G4 forming sequences with the G-runs highlighted in red.

Abbreviation	Sequence	Conformation (K ⁺)
HTelo	A GGG TTA GGG TTA GGG TTA GGG	mixed
BCL-2	GGG CGC GGG AGGAAG GGG CG GG	mixed
22CTA	A GGG CTA GGG CTA GGG CTA GGG	anti-parallel
HRAS-1	TC GGG TTGC GGG CGCA GGG CAC GGG CG	anti-parallel
<i>c-Myc</i>	GC GGG T GGG GAG GGT GGG GAAG	parallel
26CEB	AA GGG T GGG TGTAAGTGT GGG T GGG T	parallel
c-KIT2	C GGG C GGG CGCGA GGG A GGG G	parallel
TERRA	GGG UUAG GGG UUAG GGG UUAG GGG	parallel

1.3 Biological function of G4s

With a wide array of G-quadruplex structures able to form under physiological conditions *in vitro*, the question shifts to their biological role and whether they have potential as a use-

ful therapeutic target. Whilst this question is hard to answer directly and definitively, there is now a large body of evidence for the involvement of G4 DNA in biological processes.

Beginning with the publication of the full human genome in 2003, a systematic approach has been taken to identify the genome-wide location of G4 forming sequences through theoretical and experimental techniques. Indeed, many genomes including bacterial, viral and plant have now been fully sequenced and G4 forming sequences identified and compared.^[39–41] Bioinformatic approaches initially relied on the development of algorithms and used a relatively basic sequence with which to search. When four G-tracts separated with a short number of bases were used, this approach predicted around 370,000 potential G4 forming sequences in the human genome.^[42–44] The distribution of these sequences was not random; clusters were found in gene promoter regions, replication origins and at the telomeres. The early iterations of bioinformatic-based approaches were not without their limitations; for example, there are sequences known to form G4s which fall outside the original algorithms (such as BCL-2) and subsequent improvements have been made to reduce both false positive and negatives, such as the G4Hunter model.^[45,46] These algorithms take into account the large number of G4s which have been shown to form which were not originally identified by bioinformatic studies and can predict the likelihood of a given sequence to form a G4 structure; consequently they also predict between 2-10 fold more G4s than originally thought.

Concurrently, compelling evidence for G-quadruplex existence also emerged through the identification of G4-specific antibodies. This approach was first applied by Pluckthun and co-workers through the identification of a high-affinity single-chain antibody specific for G4 structures formed from the *Styloichia* telomeric repeat.^[47] With a K_d in the picomolar range, the antibody displayed 1000-fold selectivity for the parallel over antiparallel G4 topology; furthermore, ciliate telomeric G4 formation could be influenced through protein binding and cell cycle effects.^[48] Direct visualisation of G4 DNA in human cells was achieved by Balasubramanian and co-workers in 2013 through a nanomolar range G4 specific antibody (named BG4) developed through phage display.^[49] Through the use of a sec-

ondary and then tertiary antibody functionalised with a fluorophore, BG4 foci could be identified in fixed human cells. Furthermore, evidence that the staining was linked to G4 formation was collected through tracking changes in nuclear staining during different cell cycles or after treatment with the G4 stabilising ligand PDS. Additional experiments with BG4 have visualised G4s in cancer tissue as well as RNA G4s.^[49,50] There is a possibility that G4 structure distribution is changed through the induction of G4s by BG4 or by the cell fixing process itself; however, these results are still some of the strongest evidence of G4 existence to date. The use of small-molecule optical probes can address many of these limitations, imaging G4 DNA in its native environment and offering the potential to study G4 dynamics in real time. Some of the most successful approaches have been used to show that helicases can resolve G4 DNA in cells^[51] and that G4 formation is cell-cycle dependent and is disrupted by chemical inhibition of transcription and translation.^[52] More small-molecule optical probes are discussed in Section 1.4.3.

An important advancement for the field has been the development of *in situ* sequencing techniques. The first application of this utilised next generation sequencing alongside G4-dependent polymerase stalling (G4-seq): single-stranded human DNA was sequenced with and without the presence of G4 stabilising cations/ligands and the differences compared.^[53] As the quality of sequencing is highly affected by the formation of G4 secondary structures, the location of stalling sites indicated the presence of G4-forming sequences and over 700,000 putative, or pG4s, were identified. This number was much greater than the number initially estimated by the early algorithms; the majority of these additional pG4s were from sequences including bulges or very long loops.

Chromatin immunoprecipitation (ChIP-seq) is another technique which uses sequencing of chromatin to identify where antibodies selective for certain proteins or DNA structures are found. ChIP-seq can be used to study a more biologically representative form of DNA and therefore addresses a major limitation of G4-seq – namely that G4-seq analyses a denatured DNA sequence devoid of any other structural considerations. Indeed, when ChIP-seq was applied to fixed chromatin treated with the G4 specific antibody BG4, a sig-

nificantly reduced number of G4s were identified (10,000 in HaCaT cells and 1,000 in NHEK cells). The majority of these pG4s were in highly regulated depleted nucleosome regions, indicating that the majority of G4s are suppressed endogenously by the formation of chromatin.^[54]

1.3.1 Telomere biology

The region which contains the greatest number of G4 structures are the telomeres. During DNA replication the leading 3' strand is copied continuously by the DNA polymerase, whilst the lagging 5' strand is copied via repeated formation of shorter sequence primers known as 'Ozaki fragments'. As the last 5' primer cannot initiate replication of the DNA it is bound to, an incomplete sequence is copied (termed the End Replication Problem). In order to circumvent any DNA damage to important sections of DNA, a protection mechanism has evolved in which the chromosomes are capped by 2-15 kilobases of non-coding DNA consisting of a [TTAGGG] repeating sequence. Telomeric function is closely associated with genomic stability, ageing and cancer;^[55] telomeres shorten over human lifetimes^[56] and the addition of telomerase, the DNA polymerase which templates telomere extension, to healthy cells can extend their lifespan.^[57] In many cancer types telomerase is up-regulated, allowing cancer cells to proliferate indefinitely. Thus, the telomeres and telomerase activity are well established as important targets for anticancer therapeutics.^[58,59]

Investigation into telomere structure revealed that normal cells have a 200 nucleotide single-stranded overhang.^[60] The role of this overhang is to stop the ends of the telomeres from being recognised as double stranded break points.^[61] This is achieved through various capping mechanisms; for example, shelterin facilitates the formation of T and D-loops through six protein subunits. These subunits themselves are implicated in many other pathways including DNA damage, leading to a highly complex network of biological activity.^[62] G-Quadruplexes have been posited as another capping mechanism; the human telomeric sequence has been well studied *in vitro* and as discussed can form a variety of G4 topologies. Whilst telomeric DNA is unlikely to exist in its free single stranded form

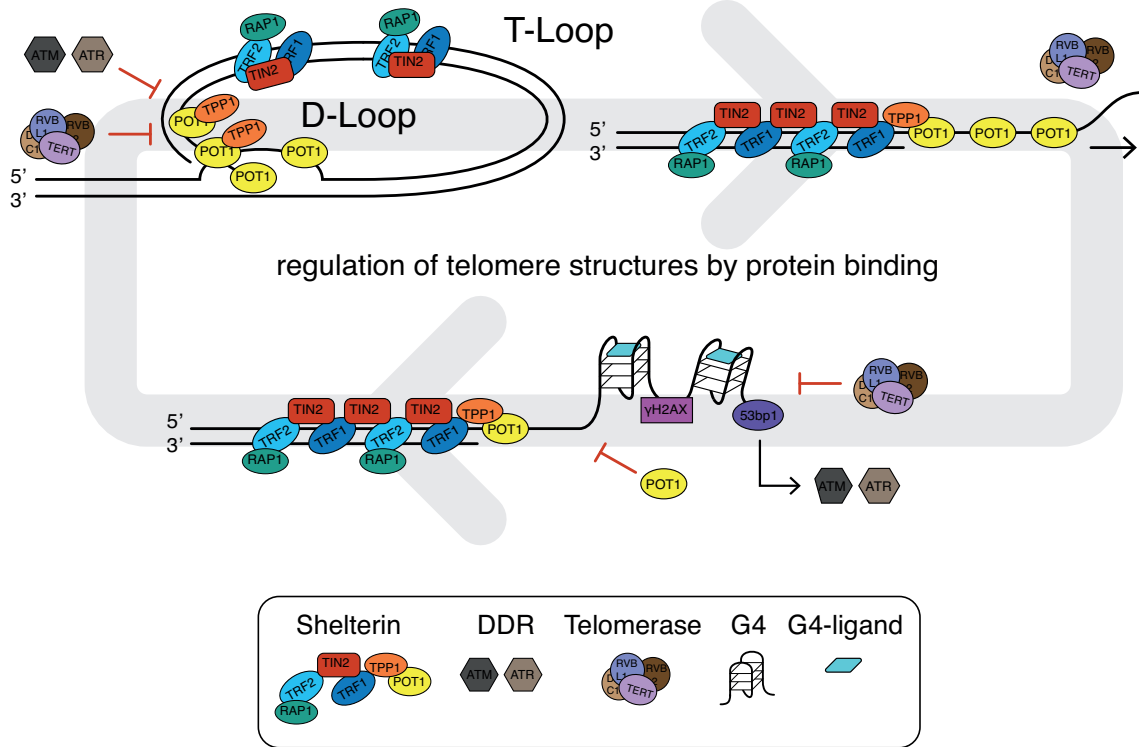


Figure 1.7 Telomere upkeep and extension: shelterin protects telomere ends from DNA damage recognition and hTERT interactions, however in cancer cells hTERT can extend telomeres, avoiding apoptosis. G4-binding ligands can reverse this effect and block hTERT.

and shelterin components can disrupt the formation of G4s,^[63–65] telomeric G4s have been shown to be able to interact with telomerase.^[66] Furthermore, G4 folding has been shown to occur within actively extending telomerase.^[67] Stabilisation of G4s with ligands can disrupt various proteins from associating with the telomeres (including telomerase), which in turn can lead to the activation of DNA damage pathways and ultimately lead to cell apoptosis and senescence in cancer cells (Figure 1.7).^[68] For a comprehensive review of all telomeric targeting G4 ligands see reference [69].

1.3.2 Gene expression

Targeting G-quadruplexes in gene promoter regions emerged as a strategy for anticancer treatment after bioinformatic techniques identified regions up to 1 kb upstream of the transcription start site (TSS) of various oncogenes which are highly enriched in pG4 sequences.^[70–72] These locations were later confirmed by the *in vitro* sequencing techniques discussed previously. Furthermore, analysis of double stranded break points (DSBs), which

are strongly correlated with the genomic instability caused by cancer and non B-DNA structures,^[73] demonstrated that DSB location can be predicted in some cancers using a G4-based algorithm.^[74] Examination of breakpoint databases has demonstrated a strong correlation between G4s, DSBs, and the expression of tumour suppressor genes.^[75] Some of the key oncogenes containing G4 structures are listed in Table 1.1, and include KRAS,^[76] HRAS,^[77] BCL-2,^[45] c-kit,^[78] and c-Myc.^[79,80] There is a greater deal of polymorphism when compared with HTelo G4 structures; loop length varies from 1 to 8 bases and each loop often has a different number of bases. Furthermore, G-tracts are not limited in either number or length.

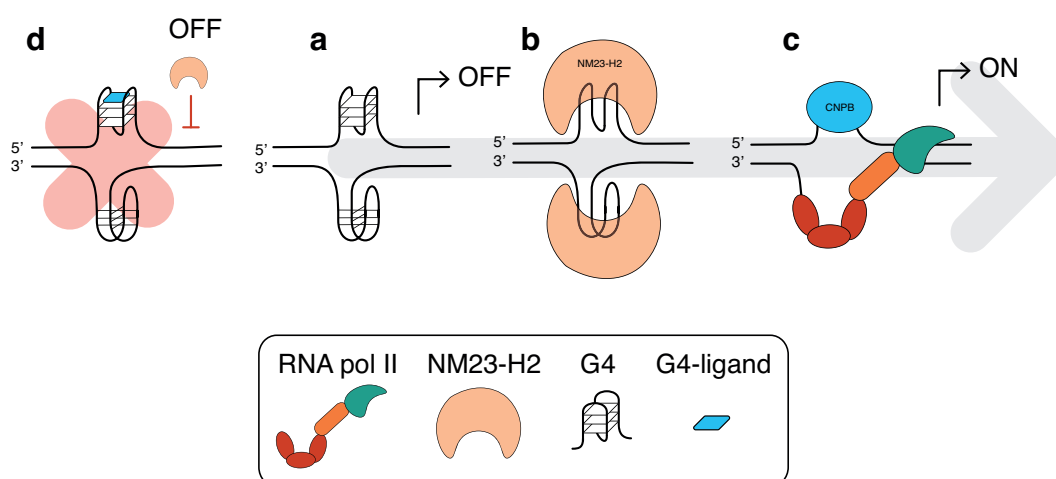


Figure 1.8 Gene regulation: (a) G4 formation in TSSs can block gene expression, (b) certain proteins can unwind G4s leading to (c) the gene can be expressed through polymerase initiation. G4 ligands can block unwinding (d).

The proposed biological role of G4s in gene promoter regions is gene regulation. For example, a study on the effect of the chemical environment in tumours demonstrated that cells from malignant tumours had lower K^+ concentrations than less aggressive cancer cell lines, and that this decrease led to lower G4 stability and a corresponding increase in the expression of *c-Myc*.^[81] Another study has used DNA damage marker analysis together with ChIP-seq to show that the expression of certain genes could be down-regulated by treatment with G4-stabilising ligands.^[78] This process is illustrated in Figure 1.8 using *c-Myc* as an example. *c-Myc* is part of the MYC family, a group of regulator and proto-oncogenes which code for transcription factors and through the expression of various transcription

factors, the genomic reach of *c-Myc* is thought to cover 15% of all genes.^[82] Normally, the formation of G-quadruplexes is suppressed in chromatin; however, during transcription, supercoiling induced by polymerase association can allow for other secondary structures such as i-motifs or G-quadruplexes to form.^[83] Further stabilisation with nucleolin proteins switch transcription off. Through association of proteins capable of unwinding G-quadruplexes (such as NM23-H2), and subsequent binding of RNA polymerase II, gene expression can be switched back on.^[84] However, the introduction of G4-stabilising ligands blocks this process and keeps gene expression switched off.^[72]

Examples of gene expression being switched on by G-quadruplex formation have also been identified. The mutations of G4-forming sequences in the promoter region of human tyrosine hydroxylase (TH) has been shown to down-regulate gene expression.^[85] Furthermore, as discussed previously, reactive oxygen species can cause the formation of abasic sites through excision base repair; this can cause the duplex G4 equilibrium to shift in the favour of G4 formation.^[86] The G-quadruplex formed can then in turn bind APE1, an activator protein AP for vascular endothelial growth factor (VEGF) or endonuclease III-like protein 1 (NTHL1) gene expression, which can cause gene expression to increase by up to 300%.^[87]

1.4 Small molecule interaction

Small molecules can be rationally designed to specifically interact with G-quadruplexes. The potential reasons for doing so are two-fold; firstly, one of the most exciting consequences of the discovery of the role G4 structures in replication, transcription, translation and telomere function is the potential for these physiological processes to be modulated through G4 specific drugs. This is particularly relevant in the cases of promotor regions of genes which encode for 'undruggable' proteins such as *c-Myc*. Secondly, in order for this approach to be successful, we need a greater understanding of the biological properties of G-quadruplexes *in vivo* and therefore the development of optical probes is also vital.

The ideal properties of a G4 targeting optical probe and a drug differ significantly; how-

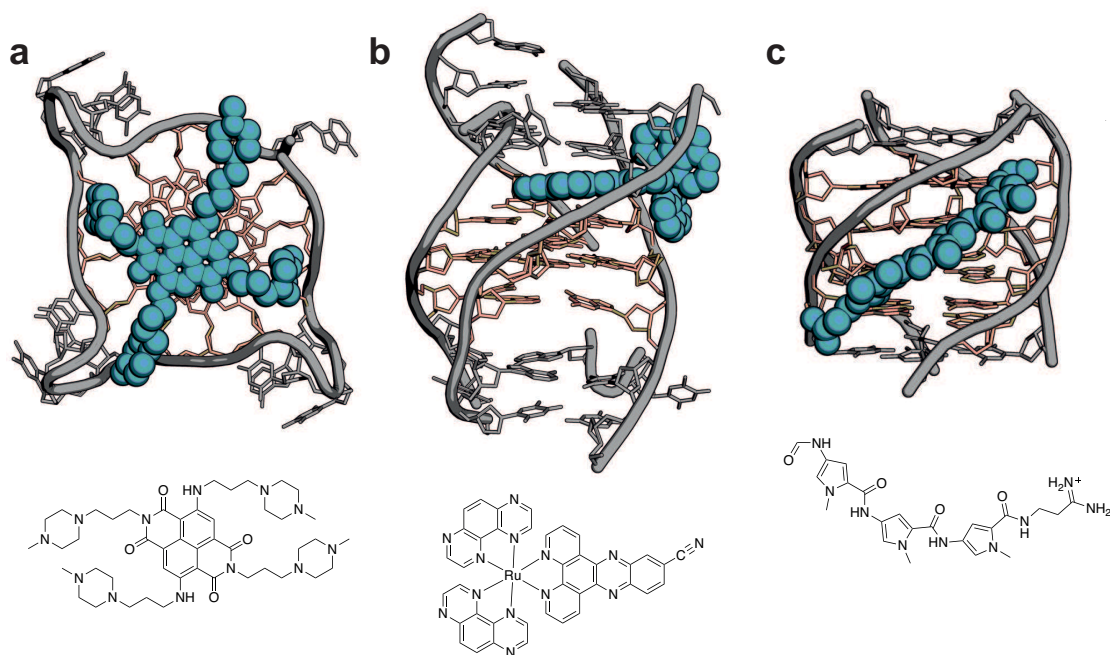


Figure 1.9 Selected examples of compounds binding to G4 DNA via different binding modes: (a) BMSG (PDB: 3T5E), (b) a ruthenium polypyridyl complex (PDB: 5LS8) and (c) groove-binding distamycin-A (PDB: 2JT7).

ever, many ligands designed for the express purpose of being good optical probes for G-quadruplexes may also display good G4 binding and stabilisation, and vice versa. This is because at a core level the design principles between the two are generally conserved; the vast majority of ligands aim to stabilise or interact with G-quadruplexes primarily through interaction with the tetrad face. The large aromatic surface is ideal for $\pi - \pi$ stacking interactions, hence good G4 ligands tend to have extended aromatic systems. To increase G4 selectivity, ligands also often have substituents which can interact with the negatively charged phosphate backbones and grooves, often tertiary amino groups (as demonstrated by G4 ligand BMSG in Figure 1.9a). These substituents also play a secondary role of providing steric bulk to minimise duplex DNA intercalation as well as increasing water solubility. Metal complexes have also emerged as highly effective G4 ligands. The metal centre can be used as a scaffold with which to construct extended aromatic systems ideal for tetrad interaction (such as ruthenium polypyridyl complexes, Figure 1.9b) and can also display interesting redox or emissive properties. In addition, in some cases the electron deficient metal centre can interact with the negatively charged ion channel. Alternative binding modes

have been identified in which G4s are stabilised solely through groove binding interactions such as in distamycin-A (Figure 1.9c); crystal structures have revealed that in some cases molecules designed to interact through $\pi - \pi$ stacking modes can also bind in this manner.^[88] The following discussion focuses on these areas; organic ligands, metal complexes and optical probes.

1.4.1 Organic G4 ligands

Many thousands of organic G4 ligands have been designed and synthesised,^[89] listed here are a combination of early and more recent ligands which have demonstrated clinical potential or are often used as controls or references in other studies (Figure 1.10). Pyridostatin (PDS) (compound **i**) is one of the most well studied G4 ligands; consisting of an aromatic core with three amino side chains, PDS has been shown impressive stabilisation of certain G4 structures.^[90] Utilising ChIP techniques by treating cells with PDS then sequencing for HDSX (a marker for DNA damage) Balasubramanian and co-workers demonstrated that sites of DNA damage matched closely with pG4 sequences.^[78] Furthermore, this overlap was highly dependent on the cell cycle phase as well as replication/transcription inhibitors, providing strong evidence that the DNA damage was G4 mediated. Recent work has shown that PDS can also prevent stalled replication forks from being restarted by homologous replication (HR), a process which normally can repair DSBs and reduce genomic instability.^[91] The authors showed that in HR defective cells, which have down-regulated levels of BRCA-1 and BRCA-2 tumour suppressor genes, PDS increased telomere fragility even in cells with DNA damage response resistance.

Neidle and co-workers recently investigated a tetra-substituted NDI derivative (MM41, compound **ii**).^[92] Characterisation of G4 binding was carried out *in vitro*, in which the ligand was shown to target G4s located in the promoter regions of bcl-2 and k-RAS genes. The compound exhibited significant *in vivo* anti-tumour activity, with dosages giving rise to an 80% tumour growth decrease. Furthermore, transcription levels of BCL-2 were reduced by up to 40%. This work is highly encouraging and signifies the potential effect of targeting G-

quadruplexes in highly networked genes. Another NDI derivative, CM-03 (compound **iii**), has been shown to be an effective inhibitor of pancreatic tumour growth and could selectively target tumours without damaging any major organs. Furthermore, upon treatment, an increase in BG4 loci and DNA damage marker co-localisation was observed.^[93]

BRACO-19 is a tri-substituted acridine derivative which also targets telomeric G4s (compound **iv**); treatment of prostate cancer cells with this ligand led to significant levels of end-to-end chromosomal fusions and inhibition of cell growth.^[94] In addition, *in vivo* experiments demonstrated that BRACO-19 was highly effective against some early stage tumours and led to decreased hTERT protein expression.^[95]

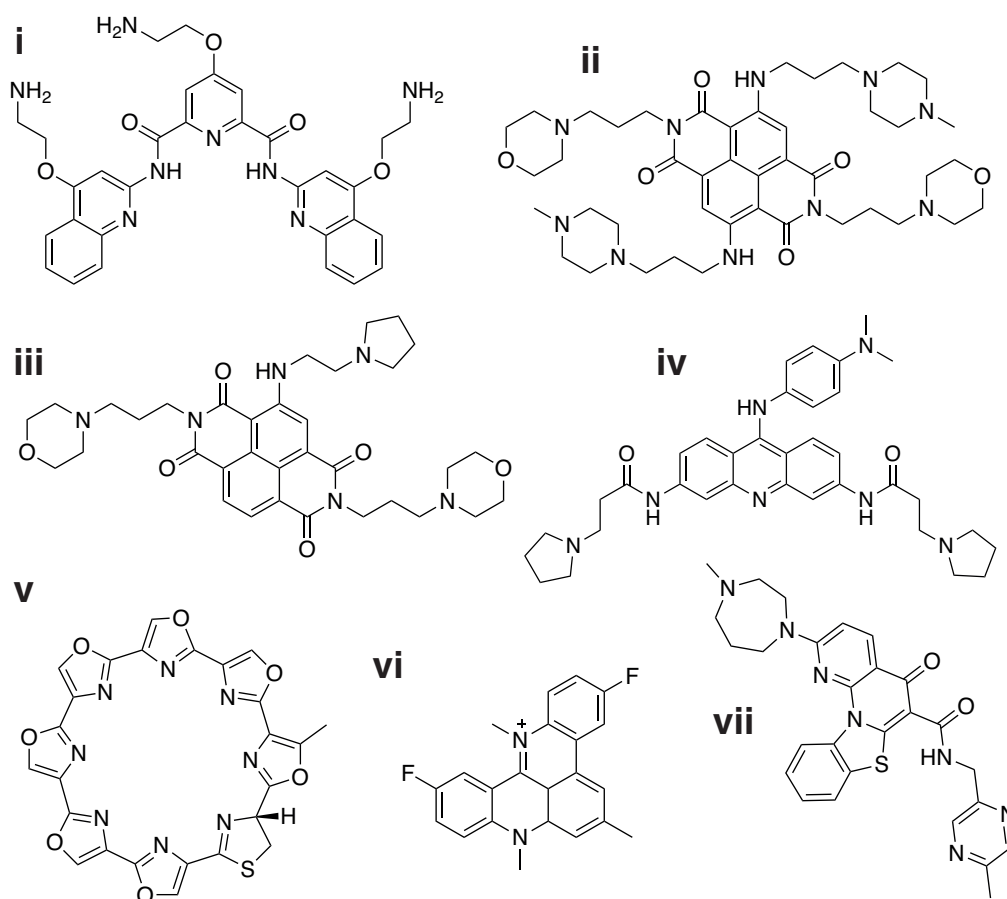


Figure 1.10 A selection of organic G4 ligands: (i) pyridostatin, (ii) MM41, (iii) CM-03, (iv) BRACO-19, (v) telomestatin, (vi) RHPS4 and (vii) CX-5461.

Telomestatin (compound **v**) is a natural product which exhibits strong telomerase inhibition and binds strongly to G-quadruplexes.^[96] In a study conducted by Brosh and co-workers, it was used to show that once cells had been treated with a small molecule

inhibitor of Werner syndrome helicase (WRN), they became highly sensitised to G-quadruplex binders or PARP inhibitors.^[97] This demonstrates the efficacy of using synergistic combinations of drugs.

RHPS4 (compound **vi**) is a ligand known to target telomeric G-quadruplexes, causing the accumulation of various DNA damage response factors and leading to tumour inhibition.^[98] Furthermore, it can trigger accumulation of PARP1 at the telomeres, which co-localises with TRF1. Biroccio and co-workers demonstrated that if PARP1 is knocked down, or PARP1 inhibitors or small interfering RNAs are introduced, tumour cell growth is inhibited through the formation of RHPS4 induced DNA breaks^[99]. This was the case even in the treatment of late stage tumours and overall survival rates were significantly improved.^[100]

To date, two G4 DNA stabilising compounds have made it to clinical trials, quarfloxin and CX-5461 (compound **vii**), both fluoroquinoline derivatives. These two drugs target ribosomal DNA through the disruption of nucleolin binding, which in turn inhibits RNA polymerase complex I, a polymerase essential for ribosomal biogenesis. Quarfloxin was shown to act effectively against a broad range of cancer cell lines and xenograft models and progressed to Phase II clinical trials (NCT00780663) before being withdrawn for poor bioavailability.^[101] CX-5461 was shown to block replication forks and induce DSBs in BRCA-1/2 deficient tumours.^[102] It has recently entered Phase II clinical trials for breast cancer (NCT02719977).

1.4.2 Metal-based G4 ligands

A significant proportion of G4 ligands developed so far are organic molecules; however, metal based systems have emerged as an alternative class of binder. Metal-organic complexes have a number of advantageous properties in the context of targeting G-quadruplexes: firstly, the central metal ion can act as a scaffold with which to assemble various organic ligands through chelation. Often the geometry of the ligands can be optimised to best interact with G4 DNA, for example through metals with a square planar geometry. Additionally, positively charged metal centres can sit above the central ion chan-

nel and interact with the electron rich oxygens in a analogous manner to the K^+/Na^+ metal cations and they can withdraw electron density from any aromatic ligands, enhancing $\pi-\pi$ interactions. Furthermore, transition metal complexes often offer interesting optical or redox properties through variable d electron counts.^[103]

The first sets of structural motifs studied were square planar metals complexed to porphyrin, salphen and terpyridine ligands alongside ruthenium polypyridyl complexes. Subsequent studies have further investigated these scaffolds as well as a many other monometallic and polymetallic structures. Metal complexes as G4 ligands have been comprehensively reviewed elsewhere,^[104,105] and as such a limited selection of different architectures will be discussed here, highlighting their diversity (Figure 1.11).

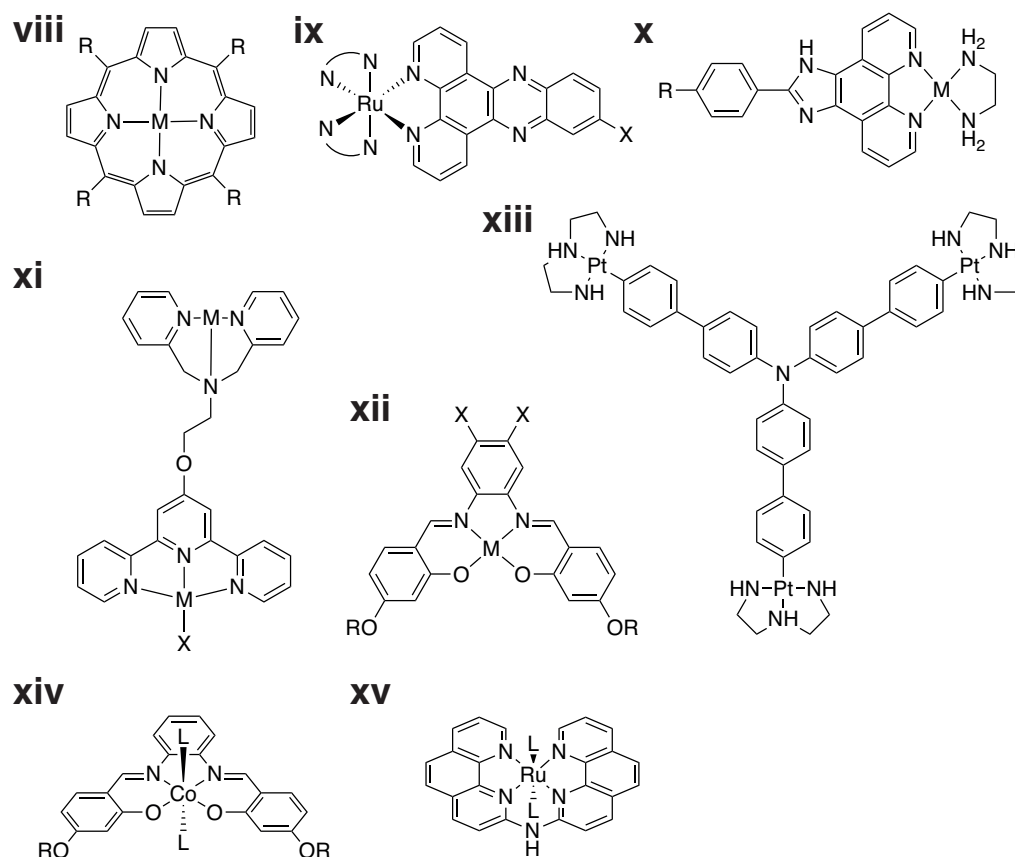


Figure 1.11 Various metal-based G4 ligand architectures: (viii) porphyrins, (ix) ruthenium polypyridyl ligands, (x) metal bipyridines, (xi) metal terpyridines, (xii) salphens, (xiii) platinum tripods, (xiv) an octahedral cobalt salphen and (xv) ruthenium bipyridines.

Porphyrins were amongst the earliest G4 ligands investigated and subsequent studies have investigated the efficacy of metal-based derivatives (compound **viii**).^[106] For exam-

ple, Yatsunyk and co-workers demonstrated that a Zn^{II} porphyrin was able to stabilise and induce G4 formation, whilst the Pt^{II} analogue destabilised G4s.^[107]

Ru^{II} based complexes have received much attention as metal based G4 ligands due to their emissive properties. For example, Monchaud and co-workers demonstrated that the use of an extended acridine-based chelating ligand (compound **ix**) led to a probe with a 330-fold switch on response in the presence of G4 DNA.^[108] Furthermore, the ligand response was highly selective for G4 over duplex DNA. More generally, the enantiomeric nature of many Ru^{II} complexes has led to some interesting examples of certain enantiomers binding well to G4 whilst the other enantiomer did not.^[109]

Phenanthroline based ligands (compound **x**) utilise a large extended aromatic system bound to a square planar Pt^{II} centre through bipyridine chelation. Systematic studies have been carried out in which the substituents and size of the aromatic surface are varied.^[110] Overall these ligands show good stabilisation of G4 structures and are able to inhibit telomerase activity.

Pt^{II} terpyridine structures are a class of metal complexes in which good selectivity for G4 over duplex binding has been demonstrated,^[111] including selectivity for *c-Myc* G4 structures.^[112] Terpyridine complexes with a second metal complex (compound **xi**) coordinated displayed significantly enhanced binding strength, proposed to be due to electrostatic/metal interactions with the phosphate backbone.^[113]

Salphen complexes consist of a tetra-dentate Schiff base ligand which stabilises a metal centre through two nitrogen and two oxygen interactions (compound **xii**). These complexes are highly stable and allow for facile functionalisation at a number of sites. They have been comprehensively investigated as G4 ligands, and display excellent G4 stabilisation.^[114–118] Our group has conducted a number of structure-activity based studies in which the metal, amino substituents and backbone groups have been varied, with excellent G4 stabilisation demonstrated.^[114] Ni^{II} and Pt^{II} salphens have been shown to be highly cytotoxic and in addition, some Pt^{II} salphens are DNA ‘switch on’ optical probes; confocal microscopy studies have shown that the cellular localisation of these complexes are highly dependent on

the cell line used.^[116] Barone and co-workers have shown that a salnaphen-like structure with a more extended aromatic system is able to selectively stabilise oncogene promoter G4 structures; in addition their complexes were cytotoxic when administered with lipofectamine in breast cancer cells.^[119] A number of salphens have been utilised for duplex DNA binding.^[120,121]

More exotic metal architectures have also been employed, as exemplified by Mao and co-workers Pt^{II}-based tripod structure (compound **xiii**).^[122] The ligand is highly selective for HTelo G4s and could induce monomeric and multimeric complexes in solutions. Interestingly, other ligands utilising such tripods have also been selective for telomeric G4s over other structures including BCL-2 and cMyc.^[123]

Recent work has utilised octahedral geometry to develop ligands in which the axial ligands interact with the central ion channel in concert with $\pi - \pi$ stacking interactions, increasing binding strength and reducing the ability to bind to DNA through intercalation. Our group recently synthesised an octahedral Co^{III}-salphen complex with axial ammonia ligands (compound **xiv**); the complex was not only highly selective for G4 over duplex DNA, but could also template the formation of G4 DNA from the unfolded sequence as well as inhibiting Pif-1 helicase from unwinding G4 structures.^[124] This data was in accordance with an earlier report of an octahedral Ru^{II} complex (compound **xv**) with axial ammonia ligands which could bind to G-quadruplexes through this interaction, with basket-type topology selectivity.^[125]

1.4.3 Optical probes for G4 DNA

An ideal G4 luminescent probe should exhibit a 'switch-on' response in the presence of a specific DNA topology, be synthetically accessible and exhibit absorption and emission profiles at biologically benign wavelengths for imaging applications. Furthermore, the probe should display relatively low G4 stabilisation in order keep perturbation to a minimum and best represent the true biological environment. Whilst many dye architectures have been investigated and reviewed, including acridines, porphyrins, triphenylmethanes

and imidazoles,^[126,127] a selection of the best performing probes is presented here (Figure 1.12). Additionally, many metal complexes (including the Ru^{II}-polypyridal and Pt^{II}-salphen complexes mentioned previously) have also been used as optical probes.^[128]

There have been a number of fluorescent G4 probes which have been used to track the dynamic behaviour of G4s through real time-live cell imaging. For example, IMT is a benzothiazole derivative (compound **xvi**) which displays a high degree of selectivity of G4 DNA and RNA structures over i-motifs, ss-DNA and ds-DNA, whilst displaying very little stabilisation.^[129] IMT was demonstrated to co-localise with the BG4 antibody, and its fluorescence output tracked during cellular progression. Tan and co-workers' coumarin conjugated probe QUMA-1 (compound **xvii**) can selectively bind to and track folding dynamics of RNA G-quadruplexes.^[130] Fluorescence of QUMA-1, an alkaloid scaffold dye, was shown to remain after treatment with DNAase but to switch off after RNAase treatment. Furthermore, fluorescence could be switched off after treatment with various known G4 binders as well as by unwinding of G4s by an RNA helicase. Some highly selective fluorescent probes have been developed which target certain G4 topologies; for example GD3 (compound **xviii**) is an acetone-based probe which preferentially binds and switches on in the presence of parallel G4 structures.^[131]

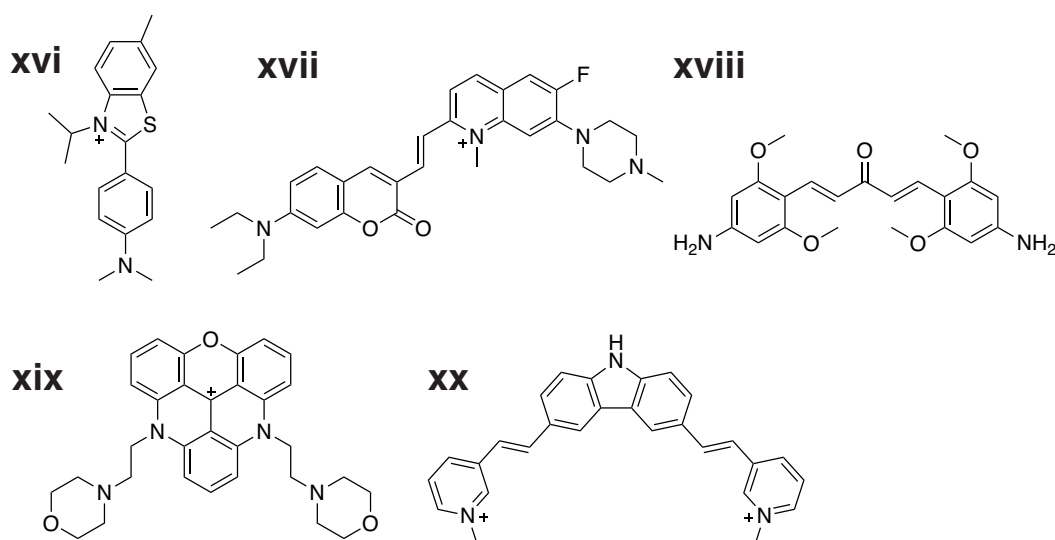


Figure 1.12 Selected examples of optical probes for G4 DNA: (**xvi**) DAOTA-M2, (**xvii**) BMVC, (**xviii**) thiazole orange, (**xix**) QUMA-1 and (**xx**) GD3.

An interesting approach with G4 optical probes has been to utilise fluorescence lifetime

differences instead of emission intensity. Lifetime dependent probes offer significant advantages over intensity based probes as the lifetime effect is concentration independent, which becomes highly important at biologically relevant ratios of G4 to duplex. For example, DAOTA-M2 (compound **xix**) is a triangulenium probe with two morpholino arm substituents which can quench the emission.^[132,133] In the presence of G4 the morpholino substituents bind to the grooves, restoring fluorescence; experiments conducted *in vitro* demonstrated that this effect was DNA topology dependent and the probe had a longer lifetime when bound to G4s than to other DNA or RNA topologies such as duplexes and single strands. In addition, the probe has ideal *in vivo* properties such as high cell permeability, low cytotoxicity, and nuclear localisation and has been used to map the location of G4 in living cells using fluorescence lifetime imaging microscopy (FLIM). More recently, the same probe was used to investigate the role that helicases play in resolving G4 DNA.^[51] BMVC (compound **xx**) is a probe based on a carbazole vinyl pyridinium scaffold which also exhibits fluorescence lifetime differences between duplex and G4 DNA.^[134] Chang and co-workers showed that this probe could detect G4s at 1000 fold lower concentrations than duplex DNA and has also been used for FLIM. BMVC derivatives with increased mitochondrial uptake have provided compelling evidence for the existence of mitochondrial G4s, again utilising variable fluorescence lifetimes; in addition, some of their BMVC derivatives could induce mitochondrial gene expression.^[135]

1.5 Approaches for controlling G4 DNA–ligand interactions

Whilst many ligands which are selective for certain G4 DNA topologies have been discovered, there remains little progress in terms of rational design for targeting one topology over another and cases of high selectivity are often due to ligand-loop interactions which are difficult to predict. There are, however, additional strategies which can be employed in order to reduce off-target effects and improve selectivity. In fact, in a biological context, there exists many other points at which to influence G4 DNA–ligand behaviour. For example, ligands can be designed which target intracellular higher order structures, such

as multimeric G4s which are specific to certain sequences. Alternatively, specificity can be improved through extracellular targeting such as cell receptors or indeed whole tumour environments.

Another very interesting approach to controlling G4 ligand binding is through the design of molecules which can exist in active and inactive forms. In these cases, a ‘caged’ G4 ligand can be activated with precise spatial or temporal resolution by application of external stimuli. The increased control gained by such systems can be vital for the study of specific biological processes and in drug delivery.

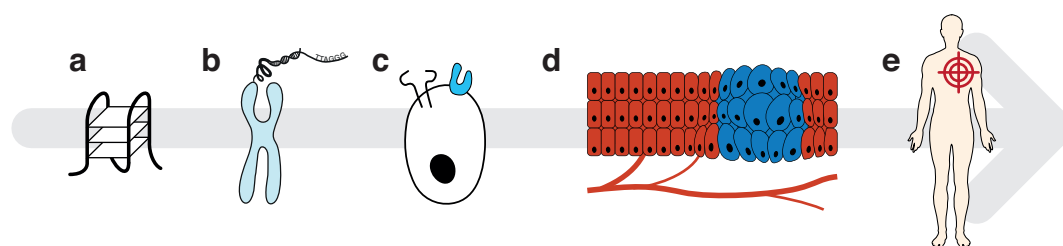


Figure 1.13 A range of points at which to improve the specificity of G4 probes: (a) topology, (b) higher order G4 structures found at the telomeres, (c) targeting cell receptors (d) specific tumour environments and (e) prodrugs capable of being triggered by external factors.

1.5.1 Targeting multimeric G4s

Compared to monomeric G4 structures, very little is known about multimeric G4s (structures which contain multiple G4 units connected by short oligonucleotide sequences). The main focus has been on multimeric telomeric G4 structures as the 200 nucleotide overhang of consisting of a [TTAGGG] repeat has the potential to form structures with contiguous G4 units. However there are also some sequences in oncogene promoter regions which have multiple adjacent G-runs such as BCL-2 and certain RNA sequences implicated in ALS also have the potential to form higher order structures.^[136] Not only might these structures be more physiologically relevant than studying single G-quadruplexes *in vitro*, but ligands designed to primarily interact with multimeric G4 interfaces might offer better selectivity for targeting specific G4s *in vivo*.

There have been a number of studies which have investigated the structural properties of telomeric multimeric G4s *in vitro*. Early work by Komiyama and co-workers utilised

atomic force microscopy (AFM) to image a 96 oligonucleotide [TTAGGG] repeat capable of forming contiguous G4 units.^[137] They showed that the structure formed consisted of four stacked G4 units and that sequences with higher order G4 formation displayed increased exonuclease resistance, in line with the hypothesis of telomeric G4 formation providing a capping function. Thermodynamic investigation of multimeric G4s revealed that multimer formation was highly sequence specific and in sequences capable of forming multimeric G-quadruplexes each unit melted separately and could destabilise others.^[138,139] Furthermore, the flanking bases also appeared to play a major role in determining stability of edge versus centre units.^[140] The topology of each unit in higher order structures is highly dependent on molecular crowding and cation; in Na⁺ buffer the topology has been shown to be antiparallel and independent of molecular crowding, whilst in K⁺ buffer, a switch from dilute to crowded conditions induced a change in topology from mixed to parallel. Interestingly, a (GGGTTA)₇GGG sequence exhibited greater stability in Na⁺ than in K⁺, in contrast to monomeric behaviour.^[141]

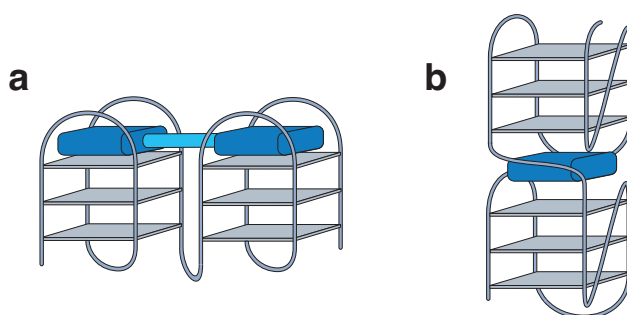


Figure 1.14 Binding modes of ligands designed to target multimeric G4s: (a) dimeric adjacent ligands and (b) cleft binding ligands.

An increasing number of studies point towards a model in which each G4 unit predominantly behaves independently, termed the ‘beads on a string’ model.^[141–143] Thermodynamic and hydration data have indicated the presence of ordered linkers between units, along with an overall unfavourable interaction between units. An AFM study which compared G-wires (in which each unit is required to be stacked) with telomeric DNA indicated that the telomeric structure formed was distinct from the G-wire structure and that the ‘beads on a string model’ is correct.^[65] In addition, they demonstrated that telomeric DNA

could form higher order G4 structures even when POT1 was bound. A study by Dotsch and co-workers utilising ^{15}N labelled oligonucleotides capable of forming multimers demonstrated that G4 units were shown to primarily exist as independent structures.^[144] Work by Saintome and co-workers focussed on whether the potential formation of multimeric G-quadruplexes could affect the ability of replication protein A (RPA), required for replication and telomere maintenance, to unwind telomeric DNA, demonstrating that increasing the number of G4 units did not effect RPA efficiency.^[145] With increasing evidence for multimeric G4s in hand, a number of research groups have also synthesised ligands which aim to selectively target these structures. They can generally be grouped into two categories; those which aim to bind at the interface between two G4 units, and those which bind across two tetrads (Figure 1.14).

Adjacent ligands

The development of dimeric ligands has generally focussed on taking well characterised monomeric G4 ligands and tethering two units together (Figure 1.15). A number of studies have been carried out with bisquinolenium ligands; Zhou and co-workers observed that certain dimeric 360A compounds (compound **xxi**) could stabilise antiparallel dimeric G4s, in addition to being selective for antiparallel topologies.^[146] Their compounds also exhibited potent anticancer effects and telomerase inhibition. A similar family of compounds was investigated by Bugat and co-workers in which their (360A)₂ compounds could displace RPA from telomeric oligonucleotides.^[147] Furthermore, investigation using single telomere length analysis (STELA) on cancer cells treated with 360A and (360A)₂ indicated the presence of ultrashort telomeres.^[148]

Zhou and co-workers sought to utilise the emissive properties of berberine-based ligands to synthesise a ‘switch on’ dimeric ligand. They synthesised a polyether-tethered dimeric berberine compound (compound **xxii**) and investigated its binding capabilities against a range of G4s. The ligand was selective for multimeric G4s, exhibiting a significant increase in fluorescence upon binding. This was ascribed to a binding mode span-

ning the plane of two adjacent G4-tetrads; the conformational rigidification of the receptor upon binding suppressing vibrational non-radiative decay pathways and causing the switch on response. A dimeric cyclic naphthalene diimide (NDI) (compound **xxiii**) synthesised by Takenaka and co-workers offered significantly improved stabilisation of dimeric G4s over the monomeric NDI; in addition, the compound offered enhanced inhibition of telomerase.^[149] Interesting work has also been carried out on 6OTD based systems (compound **xxiv**), a telemostatin analogue. A dimeric 6OTD ligand appeared to bind monomeric units by binding to both the top and bottom tetrad face and could effectively stabilise up to three contiguous G4 units.^[150] Further investigation into a similar system consisting of a 6OTD tetramer found that their probe exhibited a 40-fold greater stabilisation of multimer vs monomeric G4s. Using mechanical unfolding in optical tweezers, a binding mode in which each 6OTD unit was bound across the top of a single unit was discovered, extending up to four G4 units in a row.^[151]

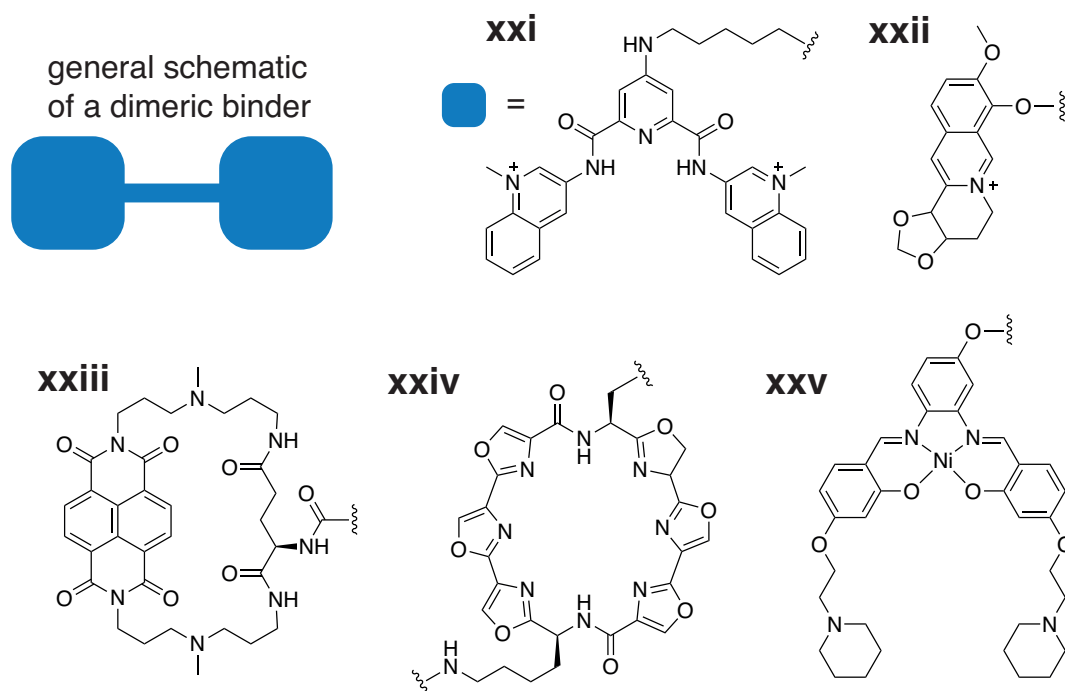


Figure 1.15 Multimeric G4 ligands featuring tethering binding motifs: (**xxi**) 360A, (**xxii**) berberine, (**xxiii**) cyclic NDI, (**xxiv**) 6OTD and (**xxv**) Ni^{II}-salphen.

Recent work by our group was aimed at synthesising a dimeric-selective ligand using the well established G4-binding Ni^{II}-salphen motif.^[152] Utilising different length polyether

linkers, a series of dinickel-salphen complexes were investigated (compound **xxv**). UV-vis titrations were carried out against K^+ stabilised mixed-type dimeric G4 and Na^+ stabilised antiparallel dimeric G4s and their monomeric analogues. The dimeric compound with the longest chain resulted in the highest calculated binding constants, comparable in strength to the monomeric salphen compound but with a high degree of selectivity for antiparallel dimeric G4. Spectroscopic studies with 2-aminopurine (a fluorescent analogue for adenine) modified sequences helped elucidate the binding mode; the results indicated that the complex interacted equally with all four tetrads in the dimeric G4 system through end stacking in a 2:1 ligand/DNA stoichiometry. These results, taken together with earlier work on the dimeric berberine complexes emphasise the importance of complementarity between ligand linker length and G4 secondary structure.

Interesting behaviour was observed with a chiral nickel metallo-supramolecular complex; one enantiomer (Ni-P) is capable of stabilising human telomeric monomeric G4s whilst the other (Ni-M) showed no monomeric G4 affinity. However, a 200-fold increase in binding constant was observed upon switching to dimeric G4.^[153] The binding mode was probed via modification of adenine to 2-aminopurine; the results indicated that Ni-M may bind via end stacking across two adjacent G quartets.

Cleft ligands

Whilst the evidence points towards multimeric G4s existing in the ‘beads on a string’ model rather than stacked, this does not preclude G4 ligands binding in the pocket between two units (Figure 1.16). TMPyP4 is a porphyrin which has shown impressive quadruplex binding properties; however, it lacks selectivity over duplex DNA. Kong and co-workers found that modifying the TMPyP4 substituents to bulkier groups gave a highly specific probe capable of selectively binding G4 over duplex DNA under crowding conditions.^[154] Furthermore, the m-isomer (compound **xxvi**) was selective for dimeric over monomeric G4s. The binding mode for m-TMPipEOPP was dependent on cavity size; a small cavity size appeared to favour an intercalative mode, whereas a large cavity caused an increase in the

binding stoichiometry ratio and a side binding 1:2 binding mode.

Hu and co-workers investigated the potential for triaryl-substituted imidazole derivative IZNP-1 (compound **xxvii**) to selectively bind dimeric G4.^[155] After proof of dimeric selectivity, further studies conducted on tumour cells which demonstrated that IZNP-1 could induce cell cycle arrest, apoptosis and senescence without affecting the transcriptional level of several non-telomeric oncogenes capable of forming monomeric G4 structures. In a similar manner to previously reported compounds, IZNP-1 appeared to derive its dimeric selectivity through an intercalated binding mode; an increase in the binding pocket size decreased dimeric G4 selectivity. Another ligand, an oxazole-triazole ligand DR4-47 (compound **xxviii**), was tested against three different dimeric G4 systems in a (1+3) topology.^[156] In both K⁺ and Na⁺ solutions, DR4-47 preferentially stabilised 2 and 3 repeat G4 sequences.

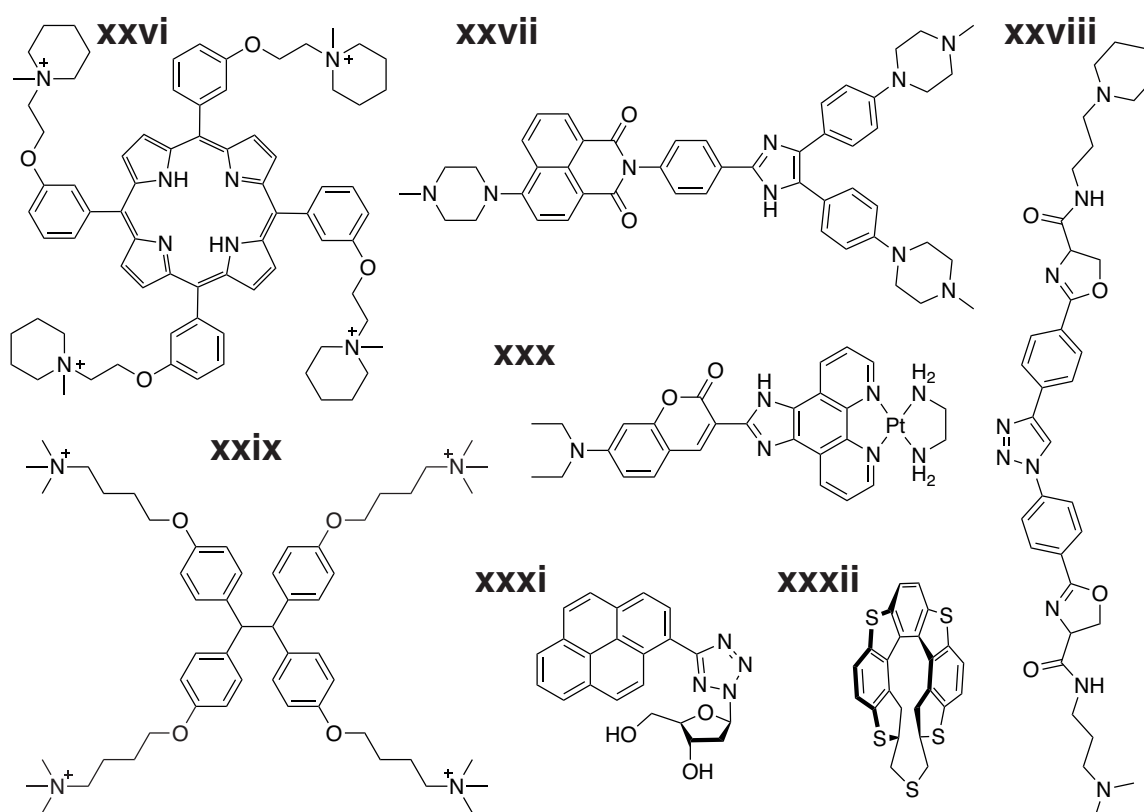


Figure 1.16 Cleft binding ligands: (**xxvi**) m-TMPipEOPP, (**xxvii**) IZNP-1, (**xxviii**) DR4-47, (**xxix**) DATPE/QATPE, (**xxx**) platinum bipyridine complex, (**xxxi**) tetrazolypyrene nucleoside probe and (**xxxii**) chiral wedge.

Zhang and co-workers investigated how tetraphenylethene dyes could function as multimeric G4 binders. Two compounds, DATPE and QATPE (compound **xxix**), comprising of

a tetraphenylethene core with two and four trimethylammonium substituents respectively, were tested against monomeric, multimeric, duplex and single-stranded DNA.^[157] DATPE was demonstrated to be selective for multimeric G4, with a significantly greater fluorescence response measured than for monomeric or duplex DNA. QATPE bound multimeric DNA more strongly than DATPE but was less selective. The difference in selectivity is proposed to derive from a decrease in electrostatic binding strength for DATPE. This interaction is not strong enough to bind to single stranded or monomeric G4 DNA but is maximised in the pocket of dimeric G4. A square planar platinum complex conjugated to a coumarin derivative was synthesised by Liang and workers (compound **xxx**), and found to stabilise mixed dimeric G4s over monomeric and duplex DNA.^[158] Again, the binding mode was found to be end stacking in the cleft pocket. Taladukar and co-workers demonstrated that their tetrazoylpyrene nucleoside probe (compound **xxxi**) exhibited an alternate binding mode, interacting with the TTA loops between two adjacent G4 units.^[159] A significant enhancement in fluorescent intensity was observed, with little effect on G4 conformation or stability. A range of chiral helicenes differing in linker length and therefore dihedral angle were tested by Sugiyama and co-workers against two dimeric G4 systems separated by two different linker lengths. The M-chiral molecule (compound **xxxii**) with the smallest dihedral angle bound selectively to dimeric G4, inhibiting telomerase activity.^[160]

1.5.2 Peptide conjugates

The inclusion of short peptide sequences is a currently under-utilised tool in G4 ligand design. Through solid phase peptide synthesis (SPPS), large libraries of peptides with varied physiochemical properties can easily be prepared and their subsequent conjugation with G4 binding motifs can allow for effective functionalisation and improved G4 specificity. However, relatively few examples exist in the literature (Figure 1.17).

Vazquez and co-workers functionalised a ruthenium complex known to bind G4 structures with an octo-arginine peptide sequence (compound **xxxiii**),^[161] which have been applied as cell-penetrating peptides to carry various cargos into cells due to their efficient

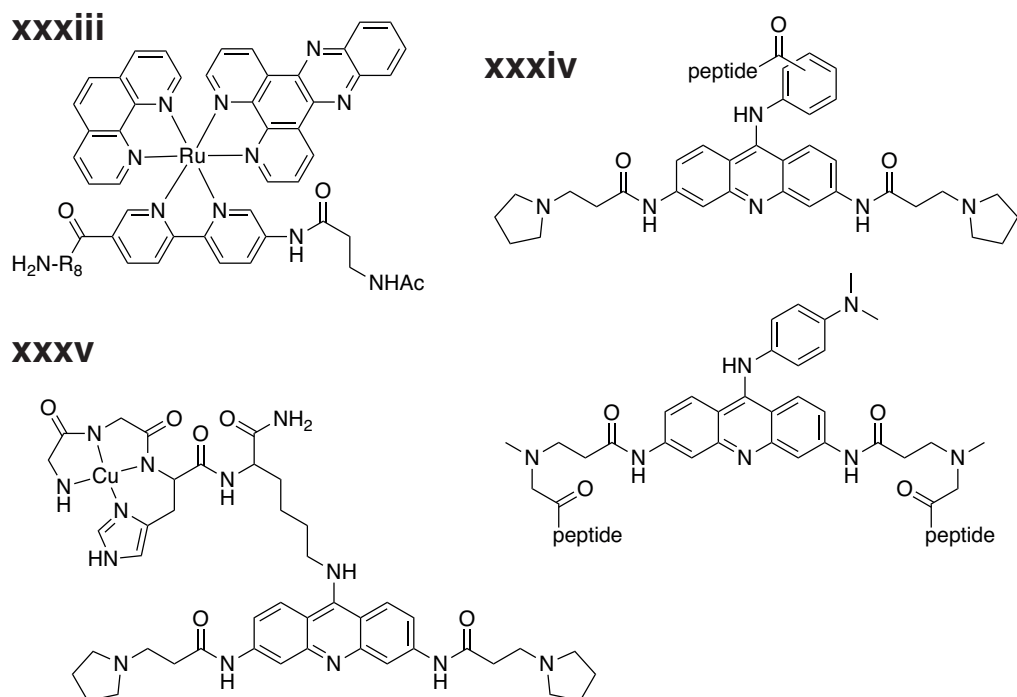


Figure 1.17 G4 ligands with conjugated peptides: (xxxiii) ruthenium polypyridyl complex with octoarginine CPP, (xxxiv) acridine based ligands conjugated with a short peptide family and (xxxv) an acridine-based ligand with an ATCUN motif conjugated.

internalisation.^[162] Furthermore, the cationic nature of the peptide sequence can often lead to improved DNA binding caused by favourable electrostatic interactions with the phosphate backbone. They demonstrated that their Ru(ddpz)(phen)(bpy) – R₈ complex displayed increased G4 binding capabilities compared with the non-peptide analogue as well as causing an inversion of G4 structure selectivity between HTelo and *c-Myc/c-kit* G4 structures. Furthermore, the compounds were cytotoxic and caused cell death with signs of apoptosis.

Balasubramanian and co-workers have investigated the effects of conjugating various peptides to an acridine binding motif based on BRACO-19. Utilising earlier work in which they used dynamic combinatorial chemistry to select for short peptides displaying G4 binding ability,^[163] they synthesised three distinct acridine–peptide families with varying peptide position (compounds xxxiv) and xxxv).^[164] Even the conjugation of a single peptide was able to improve G4 binding which, through molecular modelling, was hypothesised to be due to a synergistic effect between tetrad–acridine and peptide–groove interactions. In

addition, a number of compounds displayed good selectivity for N-Ras G4 DNA over HTelo G4. The acridine binding motif has also been used by Cowan and co-workers to synthesise a bifunctional G4 ligand capable of selectively cleaving telomeric G4 DNA.^[165] Through conjugation of an amino terminal copper nickel binding motif (a metal-binding site found at the N-terminus of some proteins)^[166] to an acridine unit (compound **xxxv**) they were able to deliver the redox active metallopeptide moiety close to telomeric DNA, promoting selective cleavage. This mechanism was able to cause significant telomere shorting and inhibition of cancer cell growth.

1.5.3 Triggerable ligands

A small number of small molecules have been synthesised in which the ability for the ligand to bind to G4 DNA is initially blocked; upon exposure to an external trigger, a form capable of stabilising G4 DNA is activated. The aim of these probes has included developing nano-devices (Figure 1.18) as well as systems in which ligand induced G4 formation is controlled to produce prodrugs with reduced off-site effects and improve specificity (Figure 1.19).

For example, Mergny and co-workers took the well established G4 ligand PDC and demonstrated that the ability of the ligand to bind to G4 DNA was mediated by Cu^{II}; the presence of the metal chelated through the pyridine nitrogen and two oxygen atoms caused a conformation switch from the active to inactive form (compound **xxxvi**).^[167] In a system including both the ligand and G4 DNA, this effect was compounded by Cu^{II} disruption of G4 structure; upon the addition of EDTA both of these effects were reversed and binding ability and G4 formation was restored. Zhou and co-workers have carried out a number of studies on an azobenzene derived ligand.^[168] Exploiting the differential abilities of the cis and trans isomers to induce G4 formation, they used visible light to switch between isomers and therefore cycle between folded and unfolded DNA (compound **xxxvii**). Further investigation using this ligand focused on a different approach; utilising host guest chemistry they blocked binding through the caging of the piperidinium substituents by cucur-

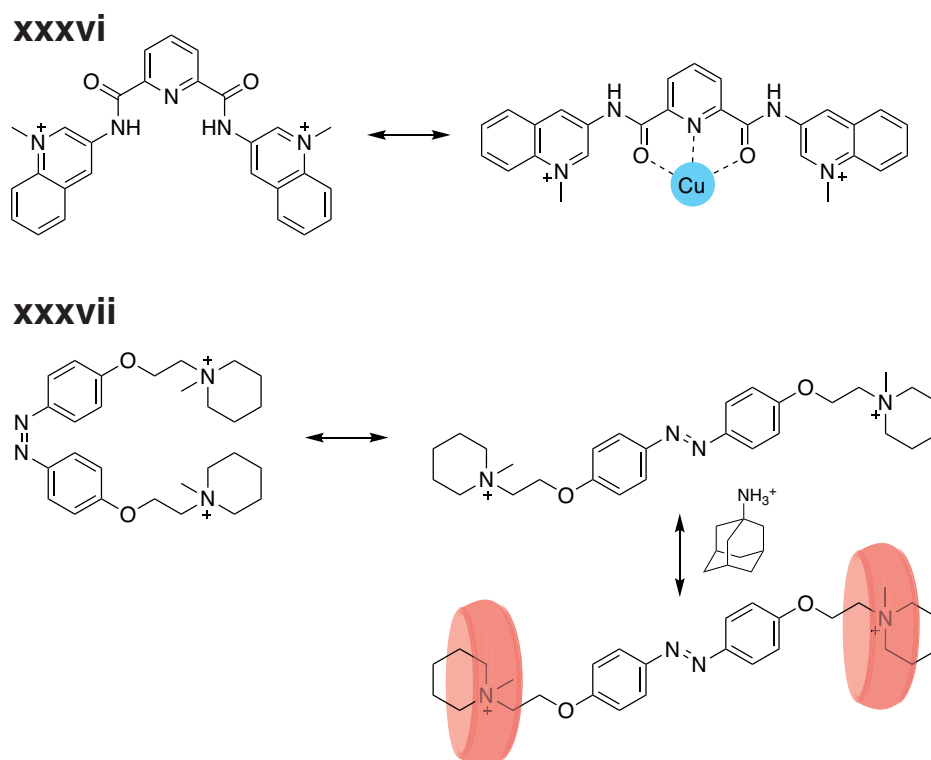


Figure 1.18 Switchable ligands used to reversibly induce G4 formation: (xxxvi) copper mediated PDC binding and (xxxvii) a switchable azobenzene derivative in which irradiation can be used to switch between E and Z isomers. Alternatively host–guest chemistry can be exploited through shielding of the quaternary amine side chains with a macrocycle, which can be displaced from the ligand using an adamantane ion with superior host–guest complementarity.

bit[7]uril (CB7) groups.^[169] In this case, the ligand was reactivated through the introduction of adamantane which has a greater complementarity with the CB7 cage, freeing the piperidinium functional groups. Again they were able to demonstrate switching between folded and unfolded G4 DNA.

A sidearm caging approach has been employed by both the Nagasawa and Balasubramaniam groups utilising a photolabile nitroveratryl (Nv) group.^[170,171] Both telemostatin (compound xxxviii) and PDS (compound xxxix) Nv analogues showed significantly reduced binding compared to the free ligand and could be quantitatively re-activated by 30 min irradiation with 365 nm light. Pleasingly, in the case of the PDS ligand, expression of several G4-containing genes was only suppressed after photo-irradiation. Finally, our group has taken a different approach, utilising the redox properties of transition metal complexes.

The oxidation of a square planar Pt^{II} salphen to an octahedral Pt^{IV} complex with chloro-axial ligands (compound **xl**) caused a dramatic decrease in G4 stabilising ability, in addition to switching off the emissive properties of the ligand.^[172] This effect could be reversed under reducing conditions such as in the presence of bio-reductants such as glutathione and ascorbic acid, paving the way for hypoxia triggered probes which are only activated in tumour cell environments.

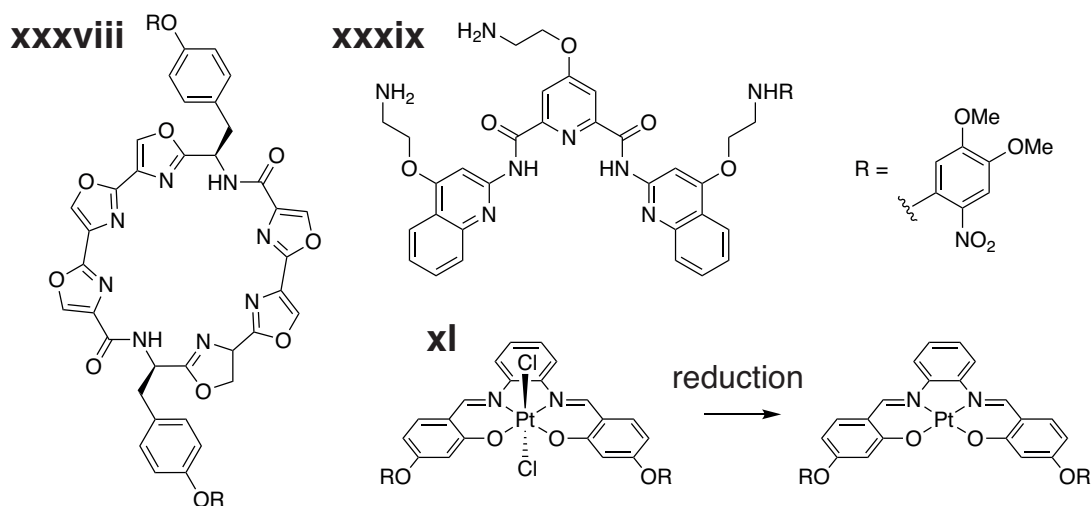


Figure 1.19 Caged G4 ligands: (**xxxviii**) telemostatin with photolabile nitroveratyl protecting groups, (**xxxix**) PDS with nitroveratyl protecting group and (**xl**) octahedral Pt^{IV} to square planar Pt^{II} transformation.

1.6 Project aims

The work described herein aimed to improve G4 DNA ligand specificity and develop new ways of caging G4 DNA ligands for triggered release. Our group has demonstrated the efficacy of Pt^{II}-salphens as G4-targeting ligands and optical probes; we therefore chose this as our starting scaffold to develop a number of different approaches (Figure 1.20). Pt-salphen derivatives suitable for click chemistry (based on high yielding cycloaddition reactions) were developed in order to expand the functionality of the system. We then applied these new Pt^{II}-salphens to target multimeric G4 DNA structures through the synthesis of a family of dimeric Pt^{II}-salphens with a range of polyether and peptide linkers (Figure 1.20a). An alternative approach was also devised in which one of the Pt^{II}-salphen units was replaced with the DNA fluorescence lifetime probe, DAOTA-M2, with the aim of having a dual-channel multimeric G4 probe (Figure 1.20b). Next, we explored how mechanically interlocked molecules could be used to modulate the G4 binding of Pt^{II}-salphens through enzymatic and photoactivated triggers (Figure 1.20c). Finally, we have developed next generation redox-activated Pt^{IV}-salphen complexes with improved stability (Figure 1.20d).

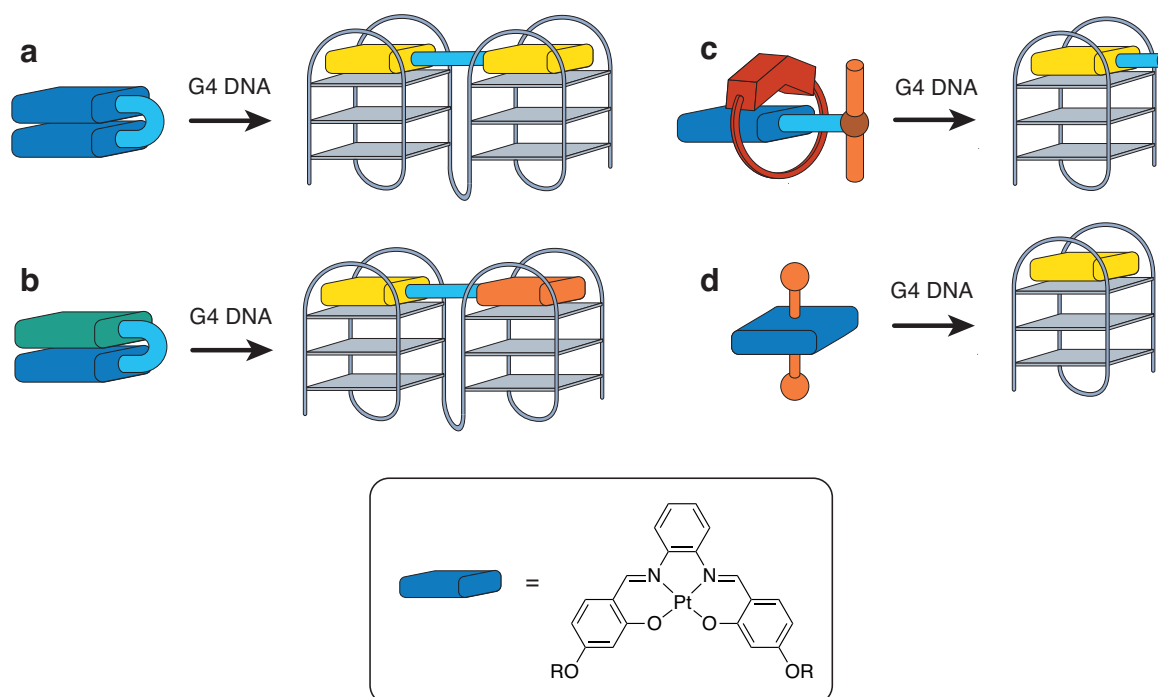


Figure 1.20 Project aims.

Chapter 2

Targeting dimeric G4 DNA

2.1 Background

As discussed in Chapter 1, many types of metal salphen complexes have been thoroughly investigated as G4 binders.^[115–118,120,121,152,172,173] This has included nickel, platinum, palladium and copper (square planar geometry), zinc (distorted trigonal bipyramidal) and vanadium (square-based pyramidal). Additionally, a wide range of amine functionalised side-chains have been tested and the size of the aromatic surface has been varied, both by extension of the diamino fragment and the side-chain fragment. Numerous studies have shown that some of the square planar family of metal salphens are particularly good G4 stabilisers and they have been demonstrated to be highly cytotoxic towards cancerous cell lines.^[116] The structural features that make salphens such effective binders are evident upon examining a Ni^{II}-salphen–G4 crystal structure; there is parallel alignment between the G4 tetrad and salphen aromatic surface indicating favourable $\pi - \pi$ interactions, the metal centre is ideally placed directly over the central ion channel and amino side chains are shown to be interacting with loops and grooves. Additionally, highlighted in red, there are sites available for additional modification.

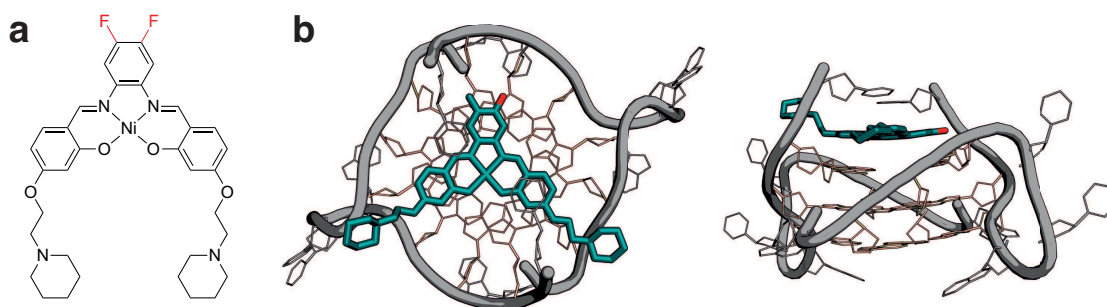


Figure 2.1 Crystal structure of a Ni^{II}-salphen with a G-quadruplex: (a) chemical structure and (b) top and side views showing interaction (PDB: 3QSC).

Our aim has been to expand the scope of metal salphen G4 ligands, with a focus on Pt^{II} -salphens. Pt^{II} -salphens are particularly attractive G4 ligands as they offer a number of highly desirable properties alongside their excellent G4 stabilisation. Firstly, the resulting compounds are emissive and can be used as optical probes for G4 structures. Secondly, they have shown appreciable photo-cytotoxicity through the generation of reactive oxygen species.^[174] Finally, the geometry of the metal centre can be modulated through control of the $\text{Pt}^{\text{II/IV}}$ redox state.^[172]

As discussed in Section 1.5.1, there are a handful G4 forming sequences which are able to form multimeric G4 structures, i.e. structures which contain two or more contiguous G4 units. These higher order structures not only might be a more physiologically relevant form of G4 DNA, but they also contain complex binding sites and pockets, which in turn could allow for the development of more specific G4 probes. In 2017 our group demonstrated that dimeric Ni^{II} -salphens (Section 1.5.1, compound **xxv**) were effective stabilisers of dimeric G4s and that the ligands were selective for dimeric over monomeric G4s, with both binding strength and selectivity influenced by the linker length between salphen units. There were, however, a number of limitations to this approach which we sought to address.

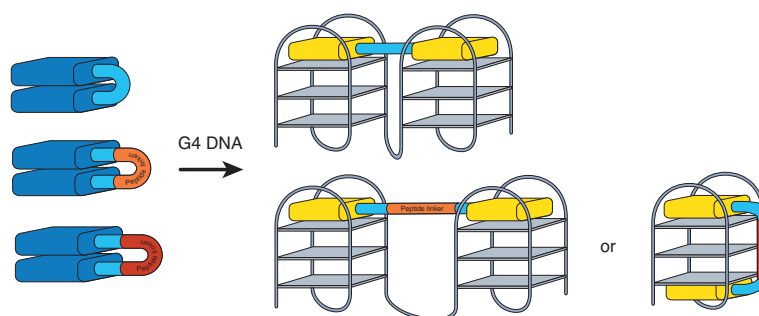


Figure 2.2 Schematic showing the switch on dimeric metal salphen complexes.

Primarily, we wished to expand this approach for Pt^{II} -salphens in order to obtain emissive analogues with the hope of imaging multimeric G4s in cells. Secondly, we were interested in improving the selectivity of these dimeric metal salphen systems. In particular, we wanted to incorporate more complex linkers into our systems and subsequently explore how this effected monomeric versus dimeric G4 versus duplex DNA binding. In order to readily access linkers with varying biophysical properties we decided to use peptides as

solid phase peptide synthesis (SPPS) can be used to access small libraries of peptides in an automated manner.

This Chapter is split into the following parts: (1) proof of principle through the synthesis of an alkyne functionalised Ni^{II}-salphen and the generation of a small library of dimeric Ni^{II}-salphen compounds; (2) synthesis of the Pt^{II} analogues and expansion to using peptides as linkers; (3) interaction of the synthesised compounds with DNA and (4) an investigation into the cellular activity of the compounds.

2.2 Synthesis

2.2.1 Probe design

A main aim we wished to achieve was to design and assemble our multimeric binders in a modular manner. Metal salphens can be synthesised through a Schiff base formation reaction. Depending on the metal required, a one-step route can be used in which the metal assists in templating Schiff base formation. Generally, this route is suitable for smaller first row transition metals such as nickel or zinc and salphen formation tends to happen rapidly. In the case of larger and more inert third row transition metals such as platinum, the Schiff base needs to be preformed, before being reacted under harsher conditions. The route previously used to synthesise the dimeric Ni^{II}-salphens involved first linking together two diaminobenzene fragments and then assembling the final compounds by a metal-templated Schiff base condensation reaction (Figure 2.3). A major limitation to this approach was that scaling up the library of compounds would involve synthesising a large range of initial precursors which would have to be compatible with each subsequent reaction condition. In addition, any future wish to investigate new linkers would always involve beginning from the first synthetic step.

We therefore aimed to synthesise an alkyne functionalised metal salphen suitable for click chemistry. Click chemistry has emerged over the last decade as an essential tool for chemists and biologists alike. Developed by Sharpless, click chemistry is defined by mild reaction conditions, high yields, and tolerance for a wide array of solvents and reaction

conditions.^[175] The most widely utilised click reaction is the copper(I)-catalysed azide-alkyne 1,3-cycloaddition (CuAAC) reaction. Not only does this reaction fill all of the criteria for click chemistry, but in addition it is bio-orthogonal, meaning it can be used for a huge variety of *in vivo* applications.^[176,177] It is therefore a highly suitable tool for increasing G4 ligand functionality and has been used across many applications such as ligand synthesis, target guided synthesis, fluorophore conjugation, PET probe development and more.^[178]

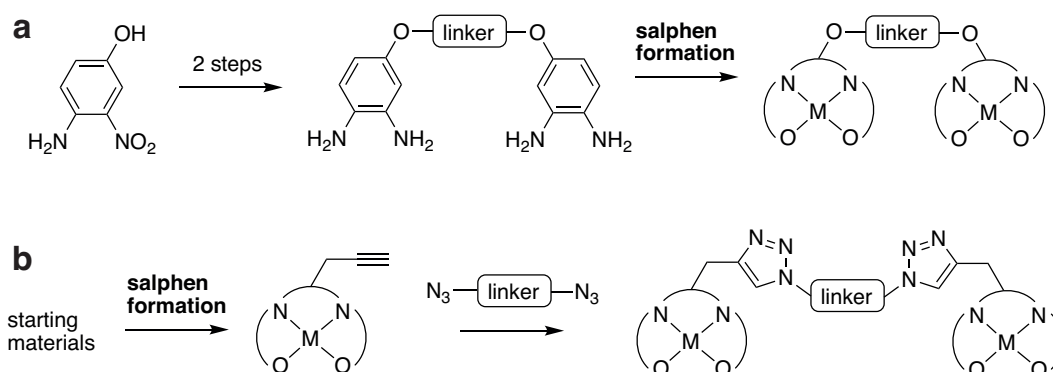


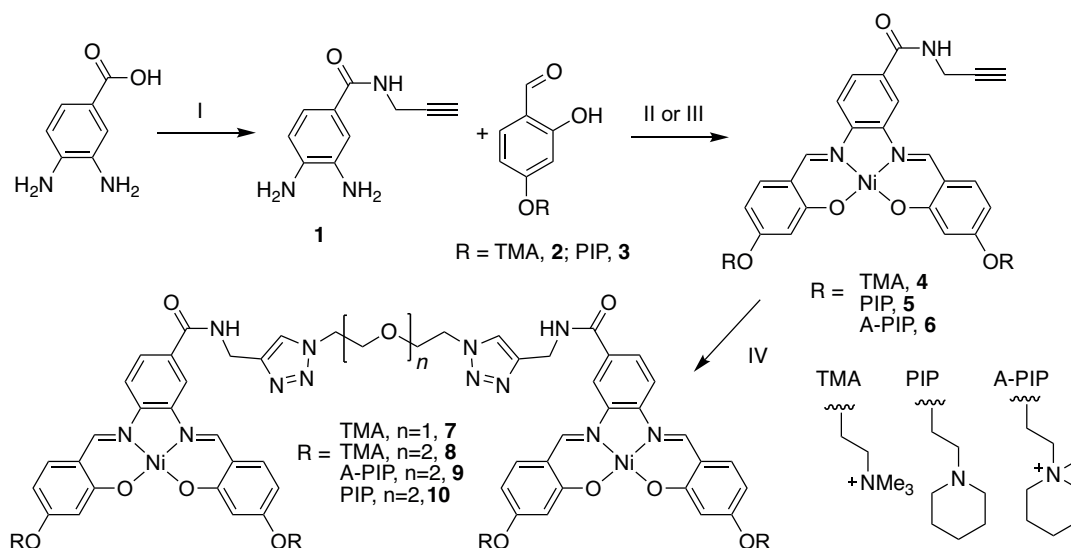
Figure 2.3 Potential routes towards dimeric metal salphens: (a) previously used route with salphen formation as the final step and (b) proposed new route utilising click chemistry.

An alkyne functionalised metal salphen would therefore allow for the facile preparation of larger libraries of compounds through the tethering of two units together through a diazide linker. Furthermore, the relatively mild conditions required for click reactions would allow us to access peptide chemistry. As Pt^{II} -salphens can be synthetically challenging to make and Ni^{II} -salphens display similar stabilisation properties but are much easier to prepare, we planned to test the efficacy of the click functionalisation using a Ni^{II} -salphen system before attempting the ultimate aim of developing a platinum-based approach.

2.2.2 Ni^{II} -salphen synthesis and characterisation

The initial synthetic targets were a family of alkyne-functionalised Ni^{II} -salphens with varying amino side chains. We aimed to test three different side chains; piperidine (PIP),^[115] alkylated piperidine (A-PIP) and trimethylammonium (TMA)^[179] in order to build a small library to establish a structure-activity relationship based on charge and steric bulk. This click-based approach was initiated by Dr Viktoria Rakers, who reported the synthesis of

the PIP and TMA derivatives at which point I joined the project. As Ni^{II}-salphens can be synthesised *via* a metal templated Schiff base condensation, precursors **1**, **2** and **3** were required (alkylation to the A-PIP side chain was carried out after salphen formation).



Scheme 2.1 Synthesis of Ni^{II} dimeric salphens: (I) propargyl amine, EDC.HCl, HOBT, NEt₃, DMF, 45 °C, 12 hrs, 52% (II) compound **1**, compound **2**, Ni(OAc)₂, MeOH, 60 °C, 12 hrs, 72% (III) compound **1**, compound **3**, Ni(OAc)₂, MeI, MeOH, 60 °C, 12 hrs, 48% (IV) compounds **4-6**, azido-2-(2-azidoethoxy)ethane/bis(2-azidoethoxy)ethane, CuSO₄·5H₂O, NaAsc, H₂O, rt, 24 hrs, 60% (**7**), 65% (**8**), 65% (**9**).

Compound **1** was formed *via* amide coupling between propargyl amine and 3,4-diamino benzoic acid. The coupling reagents (EDC·HCl and HOBT) were chosen for their lack of side product formation. Compounds **2** and **3** were synthesised according to previously established procedures;^[179,180] compound **2** was synthesised *via* a one-step S_N2 reaction between 3,5-dihydroxybenzaldehyde and chloroethylpiperidine·HCl, whilst compound **3** was synthesised *via* a two-step procedure in which 3,5-dihydroxybenzaldehyde was first reacted with dibromoethane in the presence of sodium hydrogencarbonate, followed by the addition of trimethylamine in methanol. A series of nickel templated Schiff base formations were subsequently carried out. A similar methodology was followed for all compounds in which the diamine and aldehyde precursors were combined in a 2:1 ratio in methanol and heated at 60 °C for two hours, after which 1.1 equivalents of nickel diacetate were added and the reaction stirred overnight. For salphens with TMA and PIP side chains

(4 and 5), the solvent was removed and the residue dissolved in dichloromethane, before the addition of an excess of diethyl ether caused the target compounds to precipitate out of solution. In the case for alkylated piperidine (A-PIP) compound 6, methyl iodide was added and the reaction stirred for a further 24 hours, after which a similar procedure was followed. In all cases the solid was pure enough for immediate further reaction.

The three alkyne functionalised monomeric salphens were then used to attempt to synthesise four dimeric Ni^{II}-salphen ligands. The four structures were chosen so that the effect of linker length and amino side chain could be compared. Diazide linkers with polyethylene glycol spacing units were used. A general procedure was followed; the reactants were combined in water, after which copper sulphate and ascorbic acid (1 equivalent each) were added for the *in situ* generation of copper(I). The reaction mixture was then heated overnight. Initially, the product was obtained *via* the addition of an excess of methanol, causing the dimeric product to precipitate out whilst the monomeric salphens stayed in solution. However, the salt formed (either iodo, bromo or acetate) was highly insoluble in a range of NMR suitable solvents, including DMSO and acetonitrile, which led to difficulties during spectroscopic analysis. To circumvent this issue, the counterion was switched to the hexafluorophosphate *via* anion metathesis; the dimeric salphens were dissolved in water and then added drop-wise to an aqueous solution of sodium hexafluorophosphate. Upon addition, a red solid immediately precipitated out and was filtered, washed with additional water and lyophilised. Unfortunately, this process appeared not to work well for salphen 10.

Figure 2.4 shows the ¹H NMR analysis of the dimeric Ni^{II}-salphen family. In the upper panel a representative pair of compounds is shown (monomeric salphen 4 and dimeric salphen 7). It is evident that the formation of the dimeric compound causes minimal shifts in aromatic proton environments. Importantly, the appearance of a new aromatic peak is observed at 7.86 ppm (circled in red, proton M) corresponding to the two triazole protons and a shift in the CH₂ protons adjacent to the triazole (proton L) is also observed. The lower panel shows compounds 7–10; as expected, the proton shifts are generally conserved

between compounds. For compound **10**, much broader peaks are observed along with an additional peak at 4.0 ppm indicating incomplete reaction.

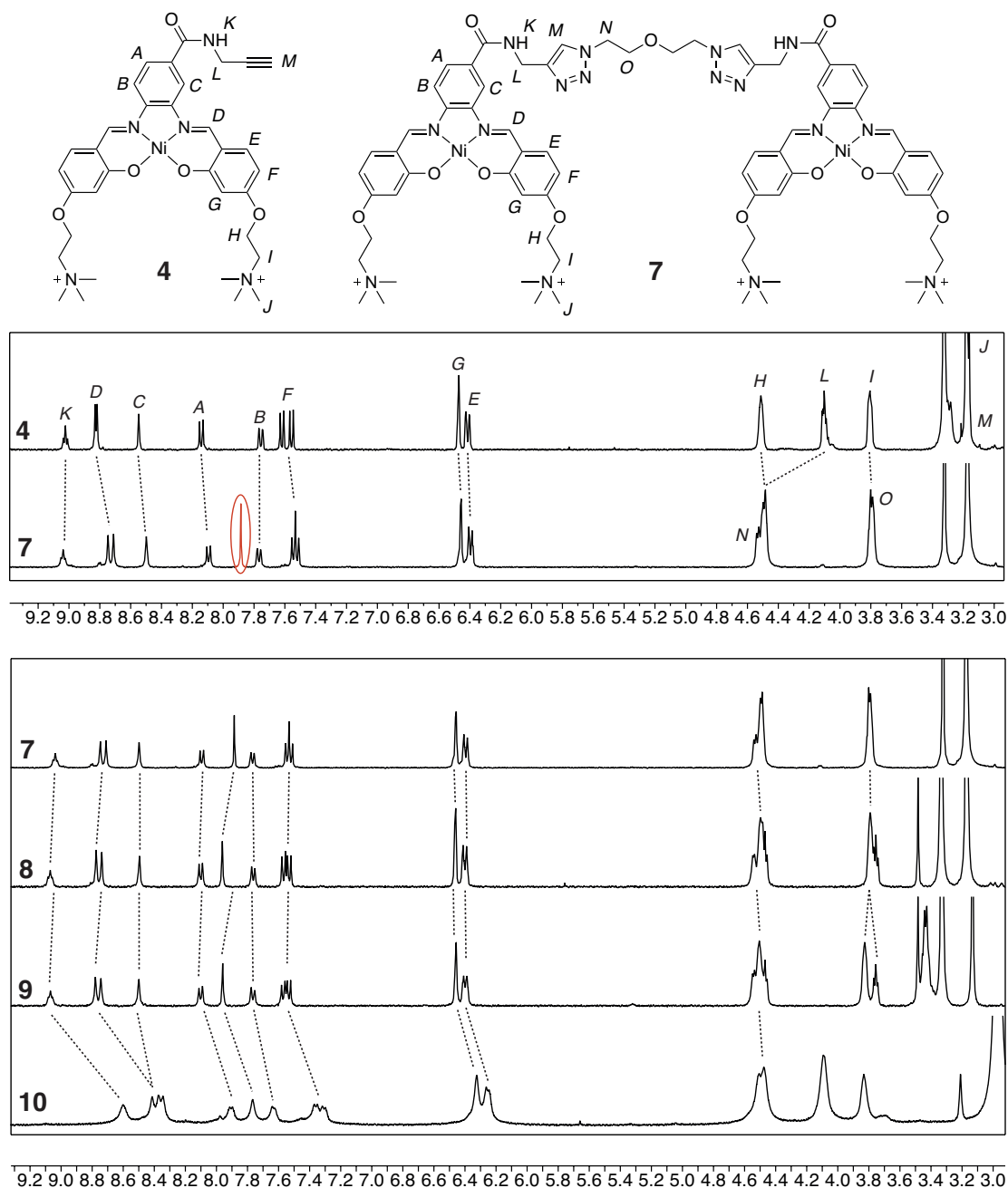


Figure 2.4 ¹H NMR analysis comparing compounds **4** and **7** (upper spectra) and compounds **7–10** (lower spectra).

The photo-physical properties of Ni^{II}-salphens were recorded. As the compounds are not emissive, just the UV-vis spectra were recorded. The concentrations of the compounds were adjusted per Ni^{II}-salphen unit. Compound **4** and **7** are shown as an example. The

compounds display the characteristic peaks of Ni^{II} -salphens, with a peak corresponding to intraligand π - π^* transitions between 300–330 nm and a second peak between 360–400 nm corresponding to charge transfer between the metal and the salphen ligand.^[115]

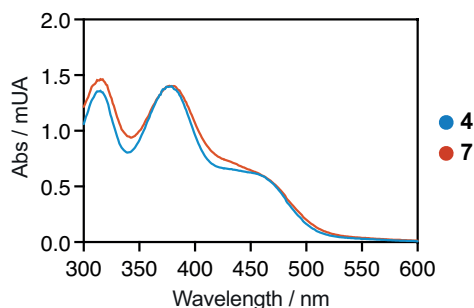


Figure 2.5 Absorbance spectra of compounds **4** and **7** ([G4 unit] = 20 μM) in water.

2.2.3 Pt^{II} -salphen synthesis and characterisation

With evidence in hand that click chemistry could be used to form dimeric salphens, the synthesis of platinum analogues of compounds **4–6** was attempted. It is not possible to use platinum as a template for the Schiff base formation due to its inertness and larger ionic radius. Therefore, a different route of that used for nickel had to be devised. A summary of the routes attempted is shown in Figure 2.6, starting from the simplest precursors; diaminobenzoic acid and compounds **2/3**. As previously discussed, diaminobenzoic acid could be functionalised with an alkyne through an amide coupling reaction with the carboxylic acid to give compound **1**. Our initial attempts focussed on the use of these precursors, utilising an established route in which the Schiff base ligand was pre-formed, after which potassium tetrachloroplatinate was added. However, when this was attempted with either diaminobenzoic acid or diamine **1**, the product formed was primarily the mono-reacted Schiff base identified via ^1H NMR, with only minuscule quantities of the desired bis product (Figure 2.6, reactions I and II). This is perhaps due to the electron withdrawing group nature of the carboxylate.

An alternative route was devised, based on literature reports in which zinc is used to template salphen formation.^[181] A trans-metallation reaction can then be carried out using platinum chloride as the zinc centre is labile and the Pt^{II} -salphen, once formed, is highly

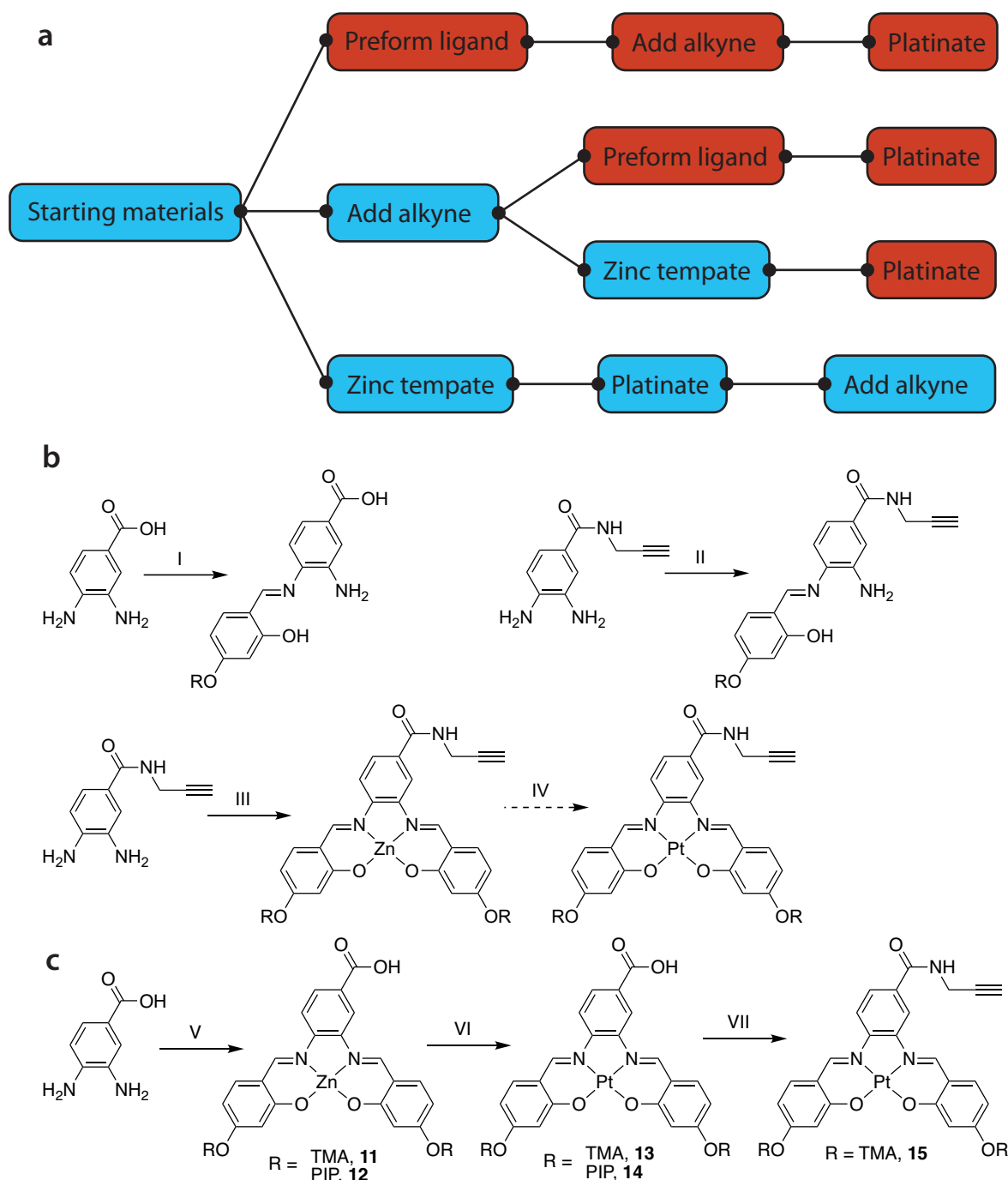
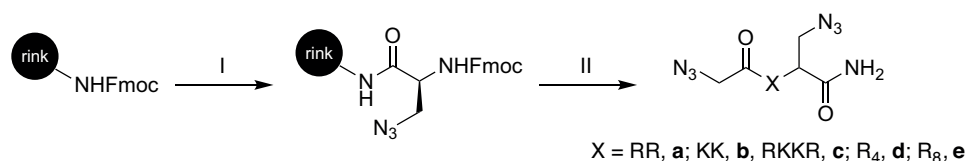


Figure 2.6 Devising a route to synthesise an alkyne functionalised Pt^{II} -salphen: (a) summary of routes tried, (b) unsuccessful reaction routes: (I/II) preforming the ligand, (III) successful Zn^{II} -salphen formation followed by unsuccessful (IV) Zn^{II} to Pt^{II} transmetalation and (c) final synthetic route: (V) compound 2, 3,4 diamino benzoic acid, $\text{Zn}(\text{OAc})_2$, DMSO, 80 °C, 2 hrs, 99%, (VI) PtCl_2 , DMSO, 80 °C, 4 days, 82% and (VII) propargyl amine, EDC·HCl, HOBT, NEt_3 , DMSO, 45 °C, 24 hrs, 95%.

thermodynamically favoured. This strategy was therefore applied with both TMA and PIP substituents to successfully synthesise both Zn^{II} -salphen analogues (Figure 2.6, reaction III). The procedure used was a modified version of that for the Ni^{II} -salphen analogues, in which the diamine and aldehyde were combined in methanol in a 2:1 ratio, followed by the addition of zinc acetate. A yellow solid was then recovered through precipitation with diethyl ether. The subsequent trans-metallation reaction was attempted in dimethylsulfoxide and the reaction heated to 80 °C for four days. After work up and ^1H NMR analysis it was evident that a mixture of products had formed. This was hypothesised to be due to the potential for platinum to catalyse cross-coupling reactions involving alkynes.

After analysis of all the potential routes it was evident that the remaining options were either to protect the alkyne before transmetallation or to add the alkyne in as the last step. It was decided that the latter would be attempted as it would involve fewer synthetic steps and protecting group compatibility would not need to be considered. In order to further optimise the synthesis, a one-pot procedure was attempted in which the zinc templated salphen formation was allowed to proceed until completion, followed by addition of platinum chloride. It was observed that the carboxylic acid functionalised zinc salphens **11** and **12** formed within two hours after heating at 80 °C. In the case of TMA functionalised compound **11**, the addition of platinum chloride caused nearly complete conversion to platinum salphen **13** which could be precipitated out using methanol, in which the Zn^{II} -salphen and starting materials were still soluble. A similar procedure was attempted for the PIP analogue **14**; unfortunately, a precipitate formed during the zinc to platinum transmetallation step which was highly insoluble and could not be analysed *via* ^1H NMR spectroscopy. We therefore decided to continue with compound **13**. Amide coupling with a slight excess of propargyl amine and standard coupling reagents gave compound **15** in excellent yield. Precipitation was achieved with an aqueous solution of sodium hexafluorophosphate.

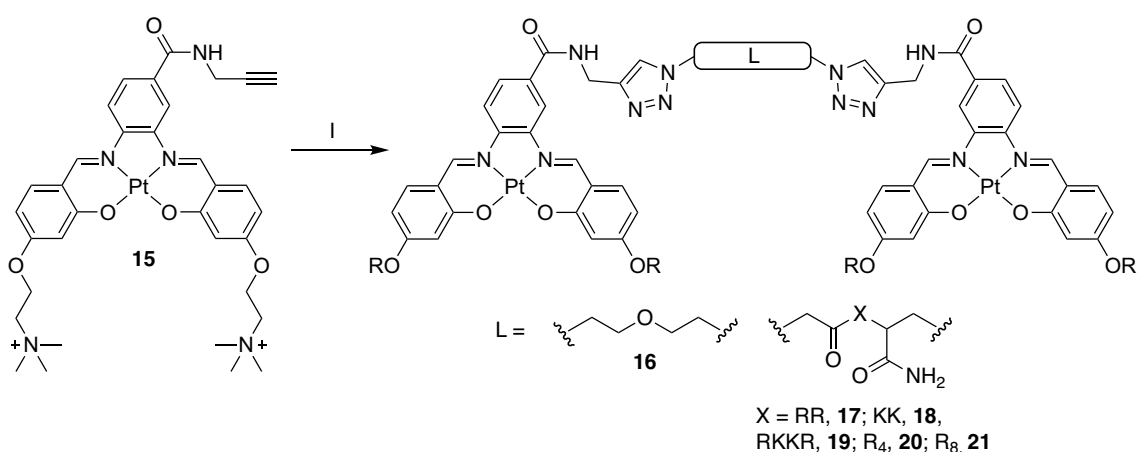
Next, in collaboration with the Vazquez group at CIQUS, Spain, we designed and synthesised a set of diazide peptide sequences consisting of a combination of arginine and ly-



Scheme 2.2 Synthesis of diazide peptides: (I) Fmoc-Rink-amide resin, Fmoc-B-aza-Ala-OH, (II) sequential addition (couple/deprotect cycles) with Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH and a final step of Fmoc-B-aza-Ala-OH followed by TFA cleavage.

sine residues designed to increase cell permeability and interact with the phosphate backbone of DNA. SPPS was used to synthesise a range of peptide based diazide linkers. An azido modified amino acid, aza-alanine, was chosen as the N terminus amino acid whilst 2-azidoacetic acid was used as the C terminus to form a diazide peptide. The middle amino acid sequence was constructed using arginine (R) and lysine (K) residues, both of which contain amino functionalised side chains which could potentially interact with the single strand of DNA linking the two G4 units. Additionally, octo-arginine sequences are known to provide improved cellular uptake. The sequences were synthesised using microwave assisted peptide synthesis and standard Fmoc protected peptides. Cleavage and de-protection of the peptide were simultaneously performed using standard conditions and the peptides were purified *via* semi-preparative HPLC.

The click reactions were then attempted with the shorter chain bis azide linker and pep-



Scheme 2.3 Synthesis of Pt^{II} dimeric salphens: (I) compound **15**, azido-2-(2-azidoethoxy)ethane or peptides **a–e**, $[\text{Cu}(\text{CH}_3\text{CN})_4] \cdot \text{PF}_6$, DMSO/water, rt, 18 hrs, yields in Methods section.

tides **a–e**. As the starting material was the hexafluorophosphate salt and therefore insoluble in water, the reaction was carried out in DMSO for compound **16** and a DMSO/water mix for **17–21** and copper(I) tetrakis acetonitrile as the copper(I) salt. As the copper is already in the 1+ oxidation state, no reducing agent was required. The reaction was allowed to proceed at room temperature overnight and near complete reaction was observed. Due to the decreased solubility of platinum salphens and additional water solubility of the peptides, no difference in the solubilities of the monomeric and dimeric products could be obtained. Purification was therefore carried out using semi-preparative HPLC. ^1H NMR analysis for compounds **15** and **16** can be seen in Figure 2.7. A difference in peak order is observed when compared to the Ni^{II} -salphen systems due to the size and electronic effects of the Pt^{II} and Ni^{II} metal centres. Much like the Ni^{II} systems, there is minimal perturbation of the aromatic environment protons upon dimerisation. The formation of the triazole peak can be seen at 7.96 ppm and there is a change in the chemical shift of the CH_2 proton directly adjacent to the triazole.

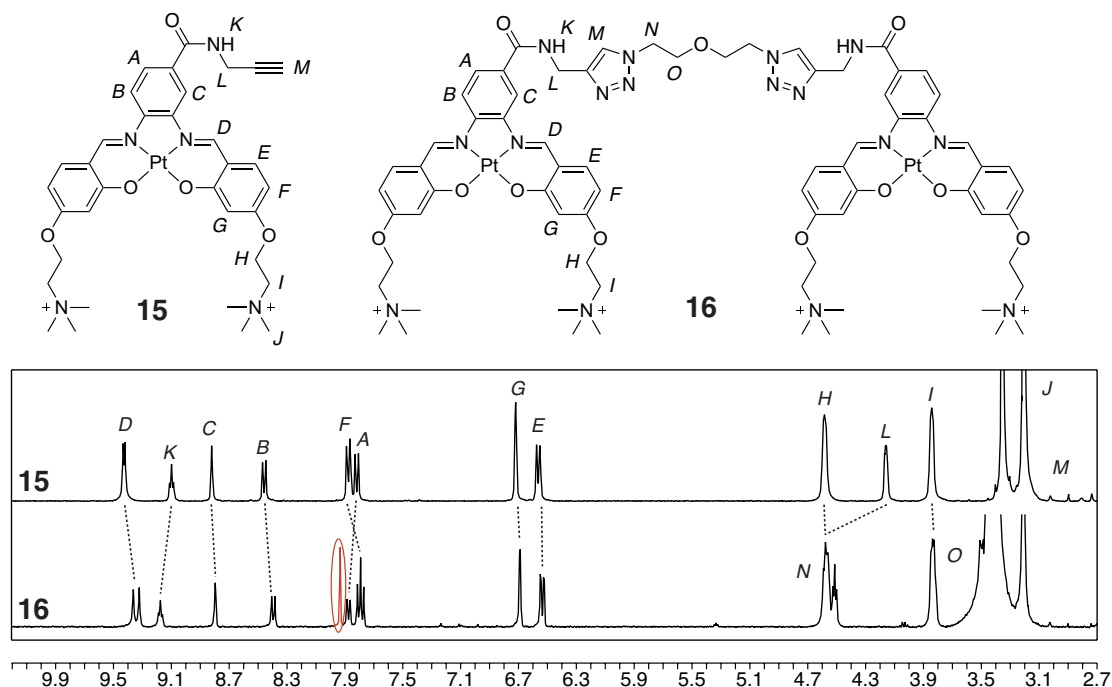


Figure 2.7 ^1H NMR analysis comparing compounds **15** and **16**.

The photo-physical properties of the Pt^{II} -salphens were subsequently measured. UV-vis and emission spectra were recorded in preparation to carry out fluorescence titrations

and are shown in Figure 2.8. The compounds display the characteristic absorption spectra of Pt^{II}-salphens: two moderate absorption bands appear at 355 nm and 325 nm assigned to intraligand transition (IL) with a third band at 426 nm, which can be assigned to a combination of metal-to-ligand charge-transfer (MLCT) [Pt(5d) $\rightarrow$ π^*] and ligand-to-ligand charge-transfer (LLCT) [(phenoxide) $\rightarrow$ π^* (imine)] transitions.^[182–184] A large Stokes shift is seen for the emission, indicative of phosphorescence. This is reported to be due to a transition between the lowest excited state, which has [L(phenoxide) $\rightarrow$ π^* (imine)] and [Pt(5d) $\rightarrow$ π^* (Schiff base)] charge-transfer character, and the singlet ground state.^[185,186] The compounds display very weak fluorescence in aqueous media due to aggregation induced quenching; emission was therefore measured in 20% DMSO in water. Both the dimeric compounds showed more effective quenching than the monomeric compound.

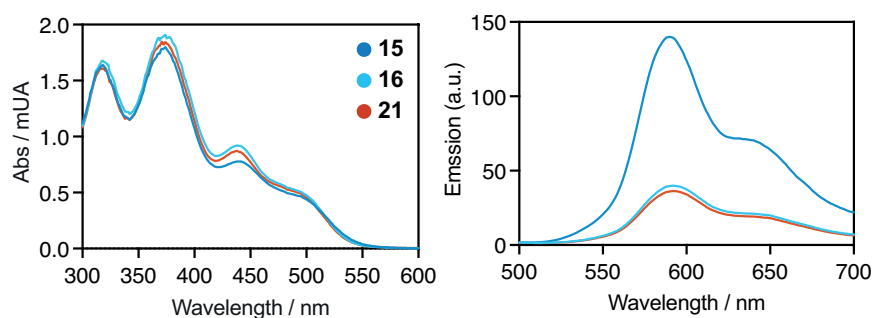
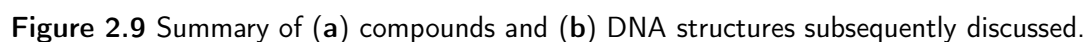


Figure 2.8 Absorbance spectra of compounds **15**, **16** and **21** [G4 unit] = 20 μ M. Emission carried out at same concentrations in water with 20% DMSO.

The full set of successfully synthesised dimeric salphens are listed in Figure 2.9. In order to evaluate binding selectivity of the dimeric metal salphens, they were compared with their monomeric counterparts. Compounds **22–24** were synthesised using analogous procedures to their alkyne functionalised counterparts. Their DNA binding properties are discussed in the subsequent Section.



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2.3.1 Circular dichroism melting

First, circular dichroism (CD) melting experiments were carried out with compounds **7–10** and their monomeric analogues **22** and **23**. The experiment was carried out *via* the combination of DNA and compound in which the ratio of salphen unit to G4 face was kept at a constant ratio of 2:1, i.e. [monomeric salphen] = 12 μM , [dimeric salphen] = 6 μM , [G1] = 6 μM and [G2Tx] = 3 μM . During the assay the temperature was increased from 25 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ and a characteristic wavelength was chosen to monitor in order to produce a melting curve.

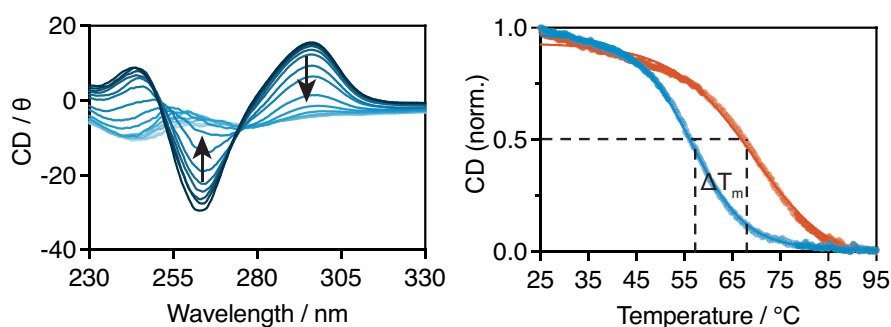


Figure 2.10 Example of how ΔT_m values have been calculated from the raw CD melting data.

An example of how ΔT_m values have been calculated is shown in Figure 2.10 in which the CD spectra has been recorded every 5 $^{\circ}\text{C}$ during a melting experiment. As the DNA unfolds, its characteristic CD spectra changes. In the case of Na^+ stabilised HTelo G4 DNA, the presence of a folded G4 structure can be tracked by following the peak at 295 nm. This data is then normalised and the data can be fitted. The experiment is then repeated in the presence of a G4 stabilising ligand. Curves are fitted using a constrained asymmetric Hill equation in which the EC50 value, or point of maximum gradient change, is taken. Next, ΔT_m values are calculated by finding the difference between the melting temperature of the G4 DNA on its own and the G4 DNA + compound.

The CD melting experiments were conducted solely with Na^+ stabilised DNA, which forms an antiparallel topology in solution. Previous work in our group has shown that if K^+ buffer is used (resulting in a hybrid G4 structure) a shift in topology midway through the melting assay is observed and therefore accurate melting temperatures cannot be obtained.

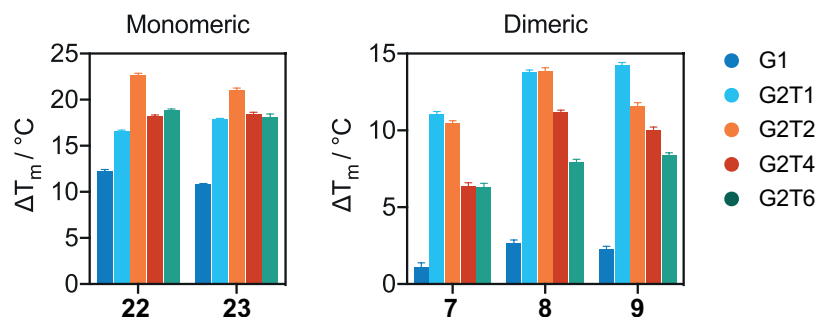


Figure 2.11 Melting temperatures calculated via CD spectroscopy of monomeric Ni^{II} -salphens **22**, **23** and dimeric Ni^{II} -salphens **7–9** with a range of dimeric HTelo (Na^+) DNA with varying linker length. The concentration of compound and DNA was varied in order to keep a 2:1 ratio of G4 unit to salphen unit. $[\text{G4 unit}] = 6 \mu\text{M}$, $[\text{salphen unit}] = 12 \mu\text{M}$. Error bars are symmetrical.

Figure 2.11 summarises the data obtained, with the values recorded in Table 2.1. First, as expected, excellent stabilisation can be seen for both monomeric salphens **22** and **23** in line with previous reports. Interestingly, both compounds stabilise dimeric G4 DNA to a higher degree than monomeric DNA. Additionally, both compounds show a slight degree of selectivity for G2T1 over the other sequences (22.5 °C and 21.0 °C respectively), indicating that perhaps there is the formation of a binding pocket between G4 units which is particularly well matched for the salphen size. However, there is generally little selectivity for dimeric G4 over monomeric G4 by these compounds. Upon salphen dimerisation, it is clear that there is a significant shift in selectivity; compounds **7–9** all show minimal stabilisation of the monomeric G1 structure (1.1 °C, 2.7 °C and 2.2 °C respectively) whilst generally retaining good stabilisation of G2T1 and G2T2 DNA and in the case of compound **7** showing ten-fold higher dimeric melting temperatures. Additionally, there is a trend in ΔT_{m} values in which lower melting temperatures were obtained for the dimeric DNA systems with longer linkers. Pleasingly, compound **7** with a single ethylene glycol spacing unit showed a steeper drop off in melting temperatures when compared with compound **8**, which contains a two spacer units. This data firstly supports the hypothesis that the dimeric salphen ligands bind across the faces of adjacent G4 units and secondly, that the longer linkers can accommodate a wider separation of G4 units. Minimal differences were observed between the compounds with TMA and A-PIP substituents.

Table 2.1 Summary of Ni^{II}-salphen CD ΔT_m values.

DNA	22	23	7	8	9
G1	12.3 $\pm$ 0.2	10.8 $\pm$ 0.2	1.1 $\pm$ 0.3	2.7 $\pm$ 0.2	2.2 $\pm$ 0.2
G2T1	16.6 $\pm$ 0.1	17.8 $\pm$ 0.1	11.0 $\pm$ 0.2	13.8 $\pm$ 0.1	14.2 $\pm$ 0.2
G2T2	22.5 $\pm$ 0.2	21.0 $\pm$ 0.2	10.5 $\pm$ 0.2	13.8 $\pm$ 0.2	11.6 $\pm$ 0.2
G2T4	18.1 $\pm$ 0.2	18.4 $\pm$ 0.2	6.3 $\pm$ 0.3	11.1 $\pm$ 0.2	10.0 $\pm$ 0.2
G2T6	18.8 $\pm$ 0.2	18.1 $\pm$ 0.3	6.2 $\pm$ 0.3	7.9 $\pm$ 0.2	8.4 $\pm$ 0.2

[G4 unit] = 6 μ M, [salphen unit] = 12 μ M

Next, the platinum salphen family of compounds including monomeric salphen **24** and dimeric compounds **16–21** were tested. It was decided that only G1, G2T1 and G2T6 would be used as there were an increased number of compounds to test. The data is summarised in Figure 2.12 with the values listed in Table 2.2. Identical conditions to that of the previous CD melting experiment were used. A melting curve could not be obtained for compound **21** as the addition of the compound to the DNA solution appeared to cause precipitation at the concentrations used.

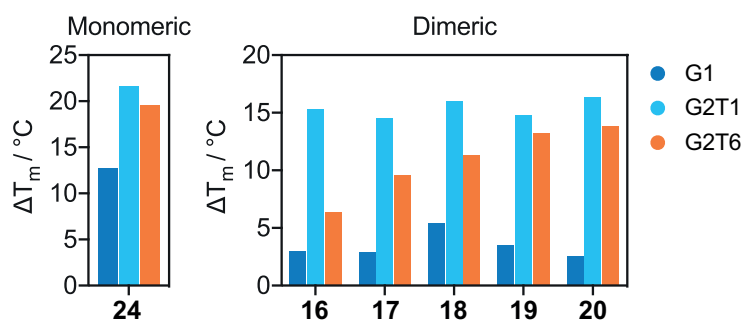


Figure 2.12 Melting temperatures calculated via CD spectroscopy of monomeric Ni^{II}-salphens **24** and dimeric Pt^{II}-salphens **16–21** with a range of dimeric HTelo (Na⁺) DNA with varying linker length. The concentration of compound and DNA was varied in order to keep a 2:1 ratio of G4 unit to salphen unit. [G4 unit] = 6 μ M, [salphen unit] = 12 μ M.

Very similar results were obtained for monomeric Pt^{II}-salphen **24** compared with its Ni^{II} analogue **22**; compound **24** effectively stabilised G1 DNA, and again a high stability was observed for G2T1 and G2T6 with little selectivity between the two. A larger discrepancy is seen between the dimeric analogues **16** and **7** (Pt^{II} and Ni^{II} respectively) in which the Pt^{II} analogue caused a greater stabilisation of both G1 (3 °C and 1.1 °C respectively) and G2T1 (15.3 °C and 11.0 °C). There are a number of additional trends across compounds **16–20**.

Firstly, it is clear that the use of peptide linkers causes a slight increase in monomeric G4 stabilisation. This is perhaps to be expected, as the additional length provided imparts the system with a greater degree of flexibility. However, whilst compound **18** with a two amino acid linker (KK) does stabilise G1 DNA by the largest amount (5.5 °C), compound **20** with a four amino acid linker (RRRR) only caused a 2.6 °C increase in melting temperature. Additionally, differences are seen between **17/18** and **19/20** when both pairs have the same length linker. This implies that the binding of dimeric compounds with peptide linkers to G1 DNA is complex and relies on an interplay of factors including sequence length and amino acids used. Whilst stabilisation of G2T1 is around 15-16 °C for the entire set of compounds, a pleasing trend is seen for G2T6 stabilisation in which increasing the linker length from a single polyethylene glycol unit to the four amino acid sequences of compounds **19** and **20** led to the ΔT_m values to more than double (6.4 °C to 13.8 °C).

Table 2.2 Summary of Pt^{II}-salphen CD ΔT_m values.

DNA	24	16	17	18	19	20
G1	12.7	3.0	2.9	5.5	3.5	2.6
G2T1	21.7	15.3	14.6	16.0	14.8	16.3
G2T6	19.6	6.4	9.6	11.3	13.3	13.8

[G4 unit] = 6 μ M, [salphen unit] = 12 μ M

2.3.2 Fluorescence titrations

Emission titrations were carried out with compounds **24** and **16–21** and G4/duplex DNA in order to obtain dissociation constants. As the experiments were carried out at room temperature, both Na⁺ and K⁺ stabilised monomeric and dimeric G4 DNA could be tested. Additionally, titrations were carried out with CT-DNA in order to evaluate the selectivity for G4 versus duplex DNA. For this series of experiments ligand concentration was kept constant at 2 μ M and the following DNA equivalents were tested: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5 (for CT-DNA 10x base pair equivalents were used, ie 1 to 50 BPE). Experiments were conducted in triplicate and binding constants were obtained by curve

fitting using Equation 1.1.

$$y = 0.5R[1/K + L + nx - [(1/K + L + nx)^2 - 4Lnx]]^{0.5} \quad (2.1)$$

Fluorescence response (y) was fitted against DNA concentration (x); R is the machine response, K is the association constant of ligand and DNA, L is the concentration of ligand and n is the number of binding sites per DNA (2 per G4 unit and 1 per CT-DNA BPE). The values of L and n were varied so that that the L was equal to the number of Pt^{II} -salphen units and n was equal to the number of G4 faces per DNA. The samples were excited at 375 nm and emission data was collected between 500-700 nm. An example of the raw data and corresponding fitted titration curve is shown in Figure 2.13.

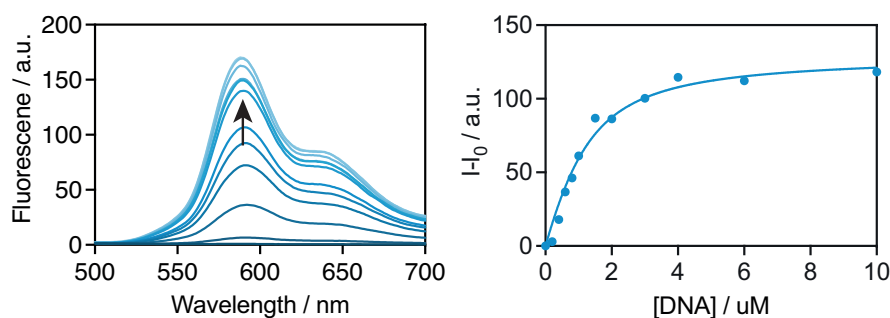


Figure 2.13 An example of the raw data collected from emission titrations and the subsequently fitted titration curve. Compound **16** with G2T1 (Na^+).

Figure 2.14 summarises the obtained results. First discussed are the experiments conducted in Na^+ buffer, in which the G4 topology formed is antiparallel. Monomeric salphen **24** showed little selectivity between Na^+ stabilised G1 and G2T1 topologies. This trend is in general agreement with the CD melting results in which Na^+ buffer was also used and higher stabilisation was seen for dimeric over monomeric G4 DNA. As discussed previously, this could potentially be caused by a sandwiched binding mode in which the salphen sits between two G1 units. For dimeric salphens **16–21** however, there are a number of differences when compared to the CD data. Dimeric salphen **16**, with the ether bridged linker, showed little selectivity between monomeric and dimeric Na^+ stabilised DNA. Upon increasing the linker length to a two amino acid sequence (RR and KK for compounds **17** and **18** respectively), larger K_a values were obtained with minimal change to the pattern

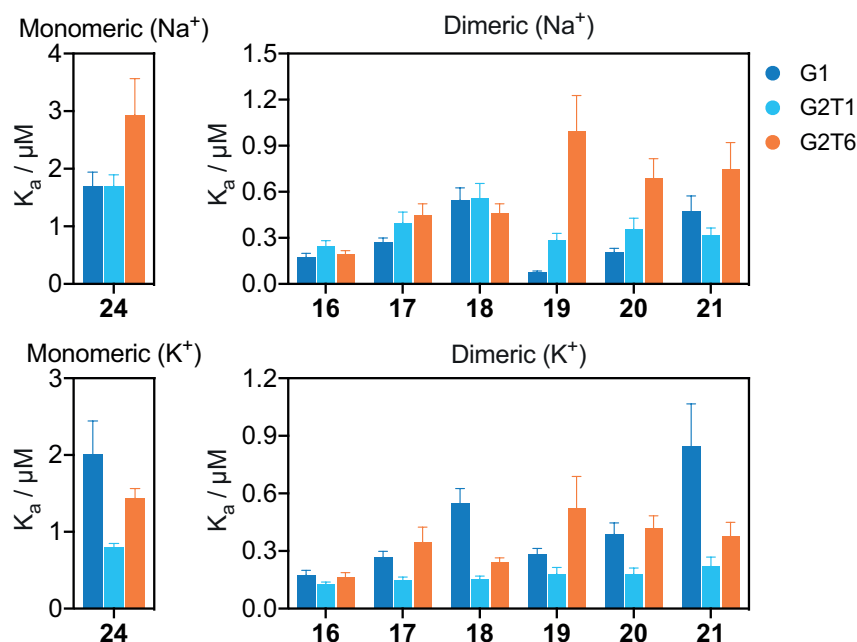


Figure 2.14 Association constants for monomeric Pt^{II}-salphen **24** and dimeric Pt^{II}-salphens **16–21** with a range of monomeric HTelo dimeric HTelo (Na⁺) and HTelo (K⁺) DNA. Excitation at 375 nm and collected at 590 nm. Error bars are symmetrical.

of selectivity. Intriguingly, compounds **19** and **20** with a four amino acid linker (RRRR and RKKR respectively) appeared to be selective for Na⁺ stabilised G2T6 over G1 DNA, with **19** in particular showing a 13-fold greater K_a value for G2T6 than G1 ($K_a = 9.91 \times 10^5 \text{ M}^{-1}$ and $K_a = 7.50 \times 10^4 \text{ M}^{-1}$ respectively). Increasing the linker length to a eight amino acid sequence (RRRRRRRR) led to a similar stabilisation of G2T6 whilst G1 stabilisation increased.

Table 2.3 Association constants extracted from Pt^{II}-salphen titration experiments.

DNA	24	16	17	18	19	20	21
G1 (Na ⁺)	1.70	0.174	0.267	0.545	0.075	0.207	0.470
G2T1 (Na ⁺)	1.70	0.245	0.397	0.555	0.286	0.357	0.315
G2T6 (Na ⁺)	2.93	0.192	0.445	0.460	0.991	0.689	0.746
G1 (K ⁺)	2.00	0.174	0.267	0.545	0.280	0.388	0.844
G2T1 (K ⁺)	0.795	0.127	0.146	0.153	0.180	0.179	0.220
G2T6 (K ⁺)	1.44	0.165	0.348	0.240	0.524	0.419	0.381

[compound] = 2 μM, [DNA] = 0.1–5 eqv. Units of K_a in μM⁻¹.

Switching the DNA topology from anti-parallel to hybrid (Na⁺ to K⁺ buffer) caused a significant change in selectivity trends. Monomeric salphen **24** now showed better binding

to G1 over G2T1 and G2T6 DNA, which is the reverse of the results obtained for the antiparallel topology. Dimeric salphens **16** and **17** had similar K_a values between topologies, with little dimeric/monomeric selectivity. Interestingly, compound **18** had a 3-fold higher K_a value for G1 DNA than G2T1 and G2T6 DNAs. Again, compound **19** showed the best selectivity for dimeric G4 DNA. Compound **20** displayed little difference in K_a values between sequences, whilst compound **21** appeared to bind significantly better to monomeric rather than dimeric G4 DNA.

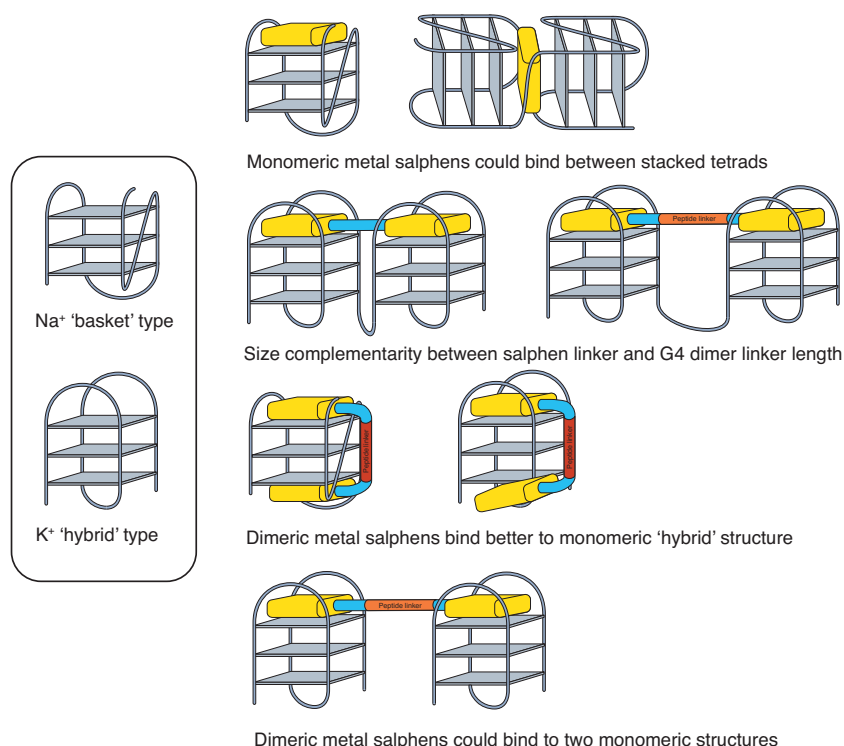


Figure 2.15 Potential binding modes.

Overall, the switch to a hybrid-type structure leads to improved monomeric binding, perhaps caused by differences in binding mode due to loop arrangements. In the case of the antiparallel solution structure, a diagonal loop crosses over one of the outer tetrads which might partially inhibit salphen binding. This effect would be significantly more pronounced for the linked salphens as the steric bulk of the linker itself could prevent the salphen aromatic surface from $\pi - \pi$ stacking with the tetrad. For the hybrid type structure, there are no diagonal loops; only propellor and lateral. This means that the second face might be more accessible for the salphen and if the linker is of sufficient length, a binding

mode could occur in which a single G4 unit is sandwiched between two salphens. Interestingly, the selectivity for dimeric or monomeric structures also depends on which amino acids are used; for example, dimeric salphen **19** with an RRRR linker showed significantly better selectivity for G2T6 over G1 than compound **20**, with an RKKR linker.

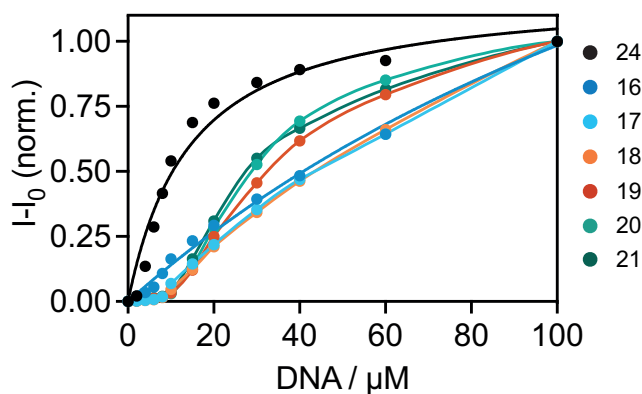


Figure 2.16 Binding curves for monomeric Pt^{II}-salphen **24** and dimeric salphens **16–21** with CT-DNA.

Next, the compounds were tested for their binding to duplex DNA, up to a maximum of 50 base pair equivalents. The results are shown in Figure 2.16. Monomeric salphen **24** showed reduced binding to duplex DNA ($K_a = 7.31 \times 10^4$) when compared to G4 DNA ($K_a = 1.5 - 3 \times 10^6$), in line with previous reports. Pleasingly, the dimeric salphens all appeared to show significantly reduced duplex DNA binding, with a ten-fold reduction in binding for compound **16** ($K_a = 5.03 \times 10^3$). Compounds **17–21** all showed a pronounced S-shape curve and binding constants could not be obtained using a 1:1 fitting model. For compounds **16–18**, the switch on effect showed no signs of saturation even higher equivalents of duplex DNA. This is potentially caused by the increased steric bulk of two tethered salphen units in which both sides of the molecule have quaternary amine side-chains, dis-favouring an intercalative binding mode. Compounds **19–21** displayed increased binding at higher equivalents of duplex DNA, perhaps caused by peptide-DNA interactions. Overall, the dimeric compounds all show better selectivity for G4 over duplex DNA than the monomeric parent compound.

2.4 Cellular studies

2.4.1 Cytotoxicity

This work was carried out by the CIQUS departmental service. Cytotoxicity studies of compounds were carried out on U2OS cell line (human osteosarcoma cells). Cells were incubated with dimeric salphens **16–21** (alongside cisplatin as control for 48 hours, before an MTS assay was used to calculate cell death. Compounds **16–20** were shown to be non cytotoxic at the highest concentration tested (100 μ M) whilst compounds **21** had an IC₅₀ value of 43 μ M. With this data in hand, cellular imaging experiments were conducted in order to confirm whether the compounds were being internalised or if the lack of cytotoxicity was due to poor compound uptake.

Table 2.4 IC₅₀ values for cisplatin and salphens **16–21** reported in μ M.

cisplatin	16	17	18	19	20	21
16	>100	>100	>100	>100	>100	43

2.4.2 Live cell imaging

Live cell images were collected using compounds **16–21**. U2OS cells were seeded at a density of 5000 cells per well and incubated for 24 hours, after which they were treated with a non-cytotoxic dose of the compounds. The cells were left for a further 24 hours and imaged using confocal microscopy. Minimal uptake was seen for **16–19**. However, for compounds **20** and **21** some uptake was observed (Figure 2.17). A series of images were taken at both 20x and 100x objectives. Whilst some uptake is clearly observed, and in many cases the nucleus of the cells shows staining, the cell morphology indicates that these cells are not particularly healthy. Additionally, given the density that the plates were seeded at, only a small subsection of cells showed staining.

Given that we would expect these Pt^{II}-salphen complexes to be cytotoxic, the cell imaging experiments and cytotoxicity results taken together provide convincing evidence that

the compounds are limited by poor cell uptake. The staining seen is therefore likely to be due to a subset of cells which were under stress, causing increased cell permeability.

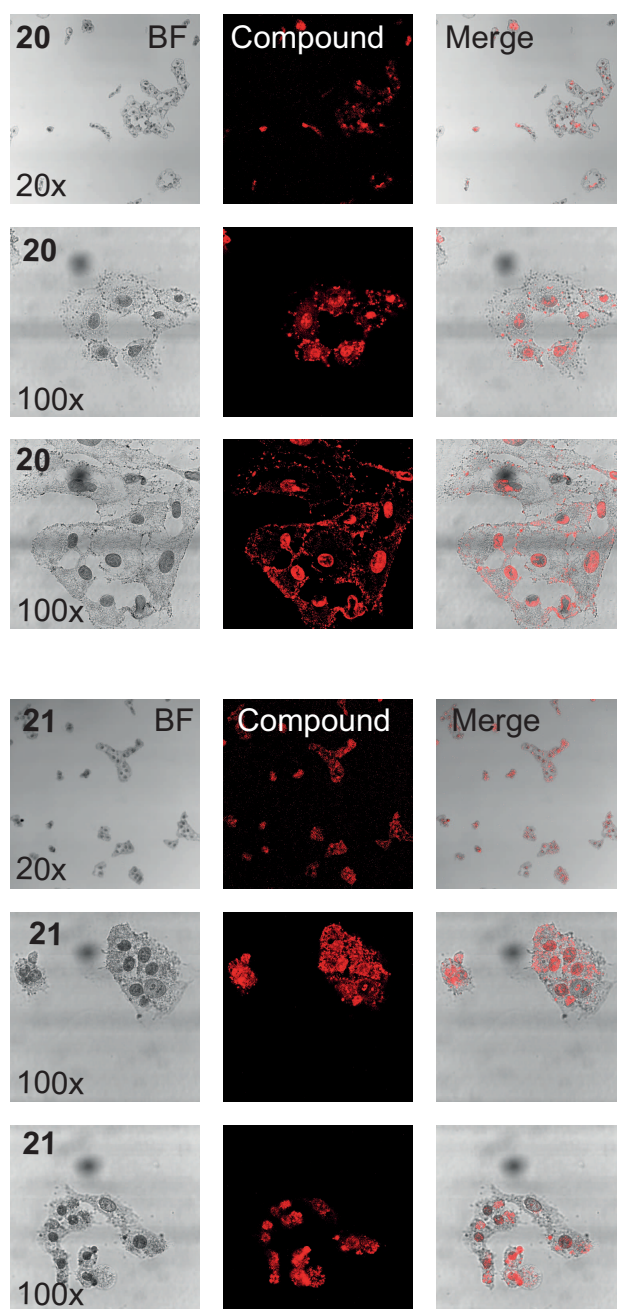


Figure 2.17 Live cell imaging for dimeric salphens **20** and **21**. Live cell fluorescence imaging experiments were collected using 10 μ M of compound ($\lambda_{\text{ex}} = 458 \text{ nm}$, $\lambda_{\text{em}} = 525\text{-}700 \text{ nm}$). All images to same scale.

2.5 Conclusions and future work

This Chapter has described the synthesis and testing of a new family of dimeric Ni^{II}- and Pt^{II}-salphens with which to target higher order G4 DNA structures. Additionally, we have expanded the scope of metal complex-peptide conjugates which target DNA and shown how click chemistry can be used to easily assemble small libraries of compounds.

Through fluorescence titrations and CD melting experiments, we have shown that the linker used to separate the metal salphen units plays a significant role in the selectivity for monomeric against dimeric G4 DNA. The results for the Ni^{II}-salphen series of compounds show that the shorter polyethylene glycol linkers show good selectivity for G2T1 over G1 DNA structures. As the linker length between G4 units was increased, the selectivity decreased. The CD melting experiments for the Pt^{II}-salphen dimeric compounds with peptide linkers demonstrated that these compounds have good selectivity for dimeric over monomeric G4 DNA. The longer more flexible peptide linkers were able to maintain selectivity for dimeric G4 structures as the linker length between G4 units was increased. The fluorescence titrations results showed a more complex picture in which a much higher variation in binding selectivity was seen for different peptide sequences and different G4 DNA topologies. For example, compound **18** showed good selectivity for G2T6 over G1 structures in Na⁺ buffer but not in K⁺. Increasing the linker length to an eight peptide sequence also appeared to lead to good G1 binding in K⁺ buffer binding, presumably due to the increased flexibility of the linker and more compact G4 structure. We were pleased to see that in all cases, the dimeric Pt^{II}-salphens showed significantly reduced binding to duplex DNA. Unfortunately, cellular experiments showed that this family of complexes was limited by poor cell uptake.

We anticipate two future paths to continue this work. Firstly, we aim to investigate whether there are any suitable macrocyclic hosts which can shield the quaternary ammonium side chains and thereby increasing cellular permeability (Figure 2.18a). As the Pt^{II}-salphen complexes showed reduced duplex DNA binding and in some cases good selectivity for multimeric G4 structures, we hope they will still be suitable for imaging multimeric

G4 structures in cells once internalised. Another avenue we hope to explore is whether the rigidity of the linker between Pt^{II} -salphen units could lead to even greater selectivity for multimeric G4 structures (Figure 2.18b).

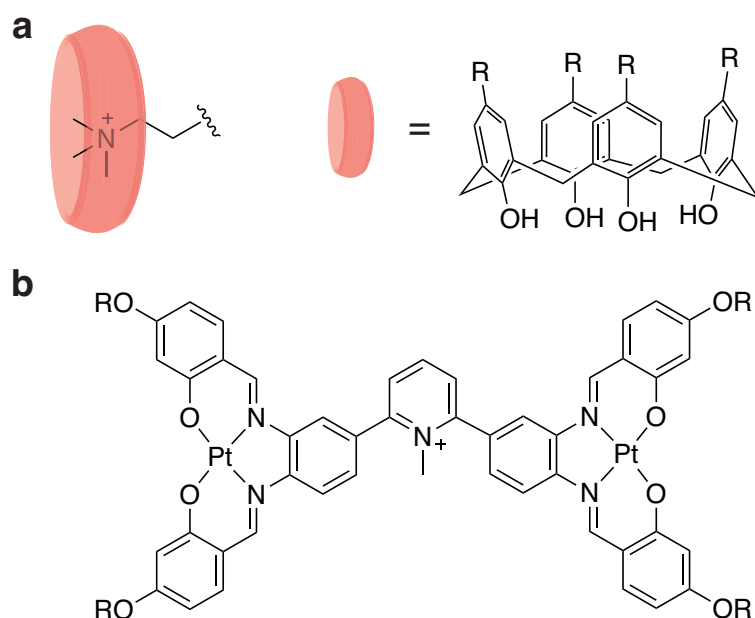


Figure 2.18 Future work to improve (a) cell permeability and (b) dimeric selectivity.

Chapter 3

A hybrid salphen-triangulenium probe

3.1 Background

In addition to the work described in the previous Chapter, we also devised an alternative approach to target multimeric G4s with the aim of forming a dual channel optical probe. Instead of using dimeric probes with repeating Pt^{II} -salphen units, we aimed to tether one Pt^{II} -salphen unit with a triangulenium-based probe. As discussed in the Section 1.3.3, our group has shown that trianguleniums are capable of discriminating G4 from duplex DNA by using fluorescence lifetime. These probes function by way of having a fluorescent core which is quenched via PET by morpholino groups. PET quenching is highly sensitive to conformation. Therefore, upon binding to duplex or quadruplex DNA, the relative position of the morpholino groups to the core leads to different lifetimes. For example, DAOTA-M2 has lifetimes when bound to duplex DNA of around 8 ns compared to 13 ns when bound to G4 DNA. The photo-physical properties of the triangulenium when interacting with DNA is highly dependent on the heteroatom bridge. For example, DAOTA (diaz-oxa-triangulenium) is a DNA 'switch on' probe, whereas ADOTA (aza-dioxa-triangulenium) undergoes slight fluorescent quenching upon binding to DNA and cannot be used as a lifetime probe.

One advantage of Pt^{II} -salphen probes is that they are phosphorescent. The much longer emission lifetime of phosphorescent probes means that they can easily be distinguished from the autofluorescence of a matrix environment. Additionally, the dramatically different lifetimes of the fluorescence and phosphorescence would potentially allow us to have two clearly separable channels if a Pt^{II} -salphen and a triangulenium were tethered together. We imagined that upon interaction with monomeric G4 DNA only one of these channels

would be activated (presumably the Pt^{II} -salphen as it has higher K_{a} values than the triangulenium). In the presence of dimeric G4 DNA both channels would be switched on, giving a unique signal. We also thought it would be important to test two linker lengths as the flexibility would be critically important in allowing each unit to interact as independently as possible. Although ADOTA had previously been shown to switch off in the presence of DNA and not provide any lifetime differences between duplex and G4 DNA, we thought that this new system was substantially different enough to warrant investigation and we imagined a scenario in which the binding of the Pt^{II} -salphen unit would force the triangulenium unit away from directly interacting with the G-quartet.

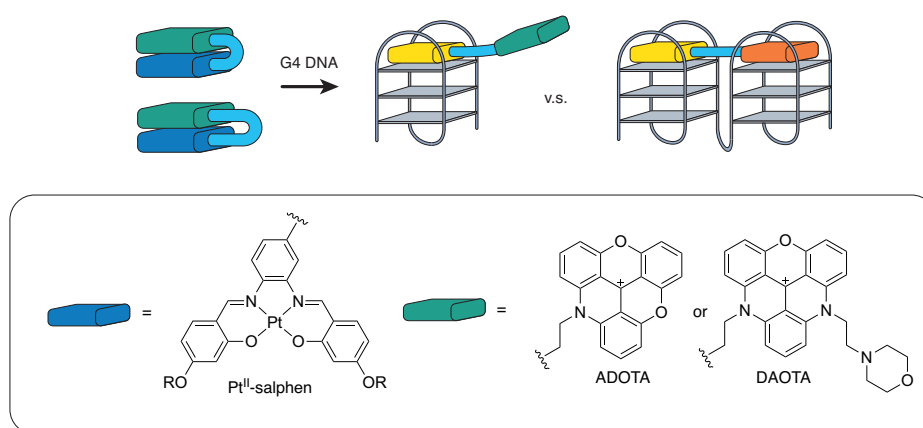
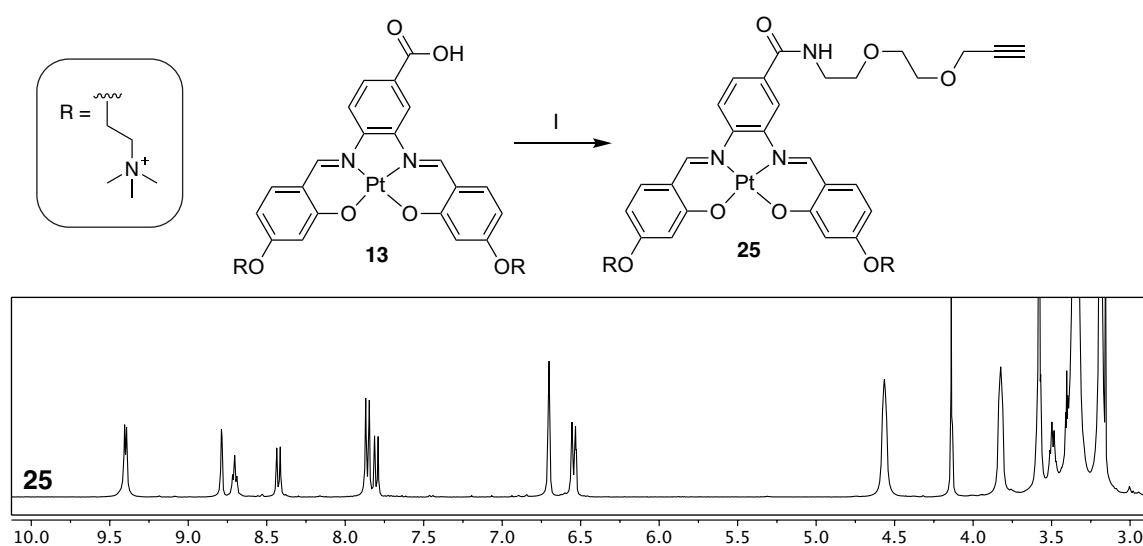


Figure 3.1 Schematic showing how a dual channel probe might be able to distinguish between monomeric and dimeric G4 DNA.

The subsequent Chapter is split into the following parts: (1) synthesis and characterisation of a new family of dimeric Pt^{II} -salphen and triangulenium compounds and (2) interaction of the compounds with DNA.

3.2 Synthesis and characterisation

The previously synthesised alkyne-functionalised Pt^{II}-salphen had a relatively short linker; therefore the initial synthetic target was an analogue with an extended linker (Scheme 3.1). Pt^{II}-salphen **25** was synthesised in an analogous manner as to before, in which Pt^{II}-salphen **13** was reacted with an amine functionalised alkyne. The ¹H NMR spectrum is shown, in which the aromatic region is broadly similar to the shorter chain analogue (Chapter 2) and the aliphatic region has additional peaks corresponding to the extended PEG linker separating the amide bond and alkyne.



Scheme 3.1 A long-linker Pt^{II}-salphen analogue: (I) propargyl amine, EDC·HCl, HOBT, NEt₃, DMSO, 45 °C, 24 hrs, 95%. Below, ¹H NMR of **25** in DMSO.

Next, synthesis of the azide ADOTA and DAOTA analogues was carried out. The synthetic targets are shown in Figure 3.2 and are direct analogues of the previously investigated ADOTA-M and DAOTA-M2 probes.

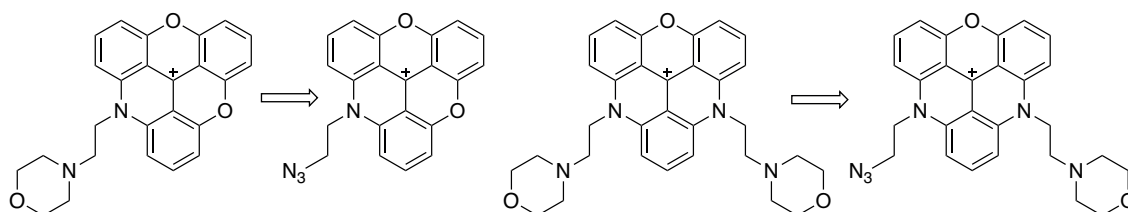
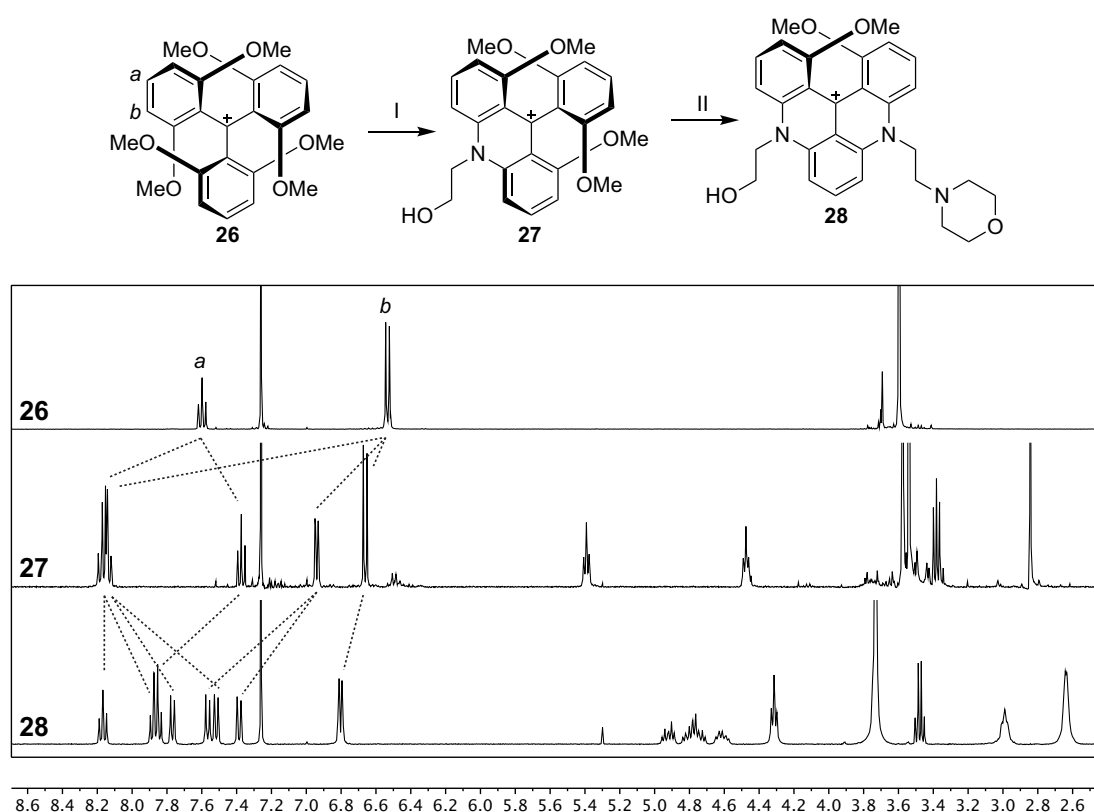


Figure 3.2 Original ADOTA-M and DAOTA-M2 probes and their azide functionalised derivatives for click chemistry.

Trianguleniums can be accessed via formation of an initial carbocation followed by successive nucleophilic aromatic substitutions. Compound **26** was formed by reaction of 1,3-dimethoxybenzene with diethyl carbonate in the presence of *n*BuLi and a catalytic quantity of tetramethylethylenediamine (TMEDA). Reaction of the resulting carbinol with tetrafluoroboric acid led to the carbenium product **26**. Product formation was tracked via disappearance of the proton *ortho* to the methoxy groups as well as downfield shift in the *para* proton, with the product displaying one triplet corresponding to proton *a* and a doublet corresponding to *b* (shown in Scheme 3.2).



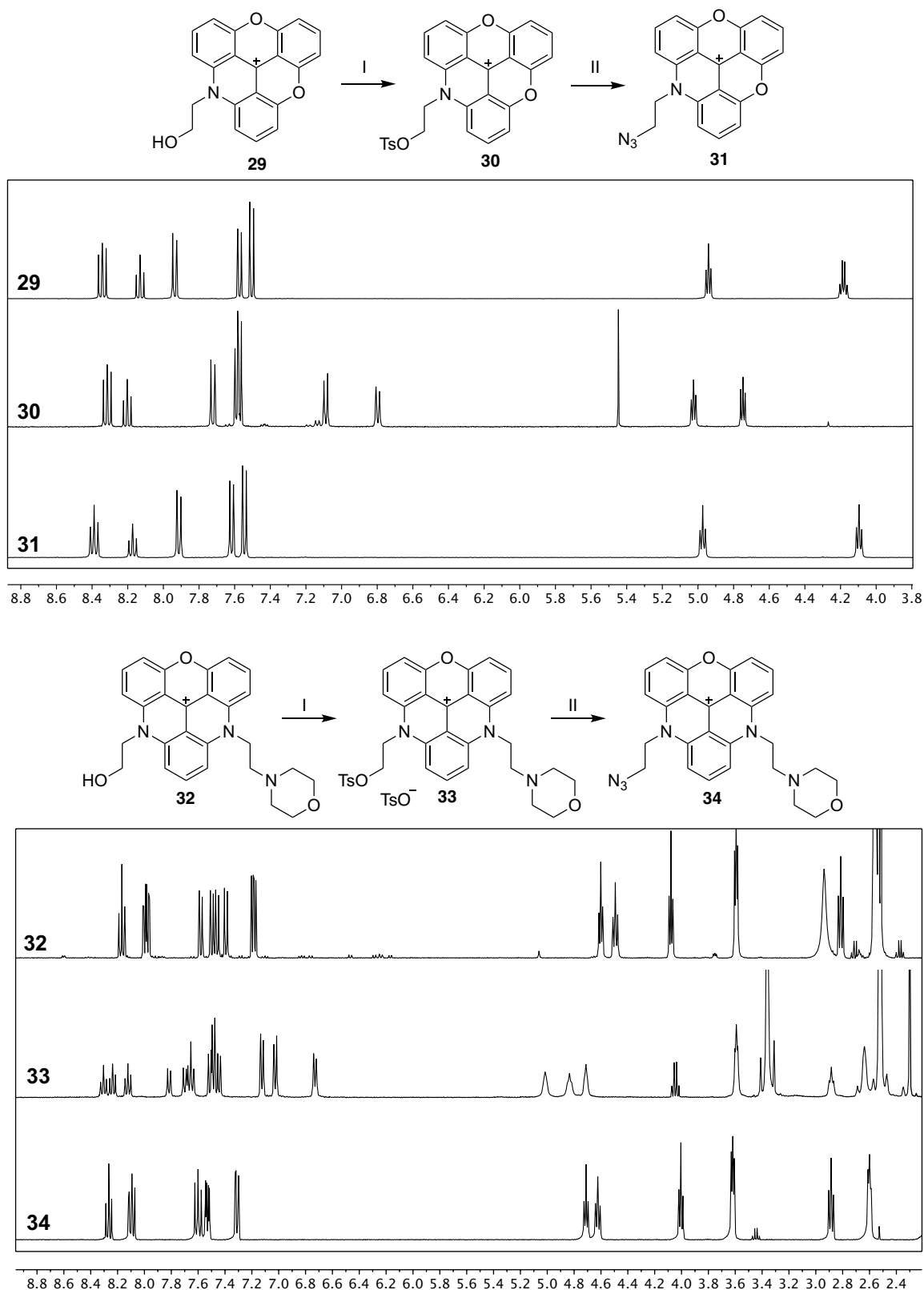
Scheme 3.2 Triangulenium precursors: (a) (I) ethanoloamine, NMP, 2 hrs, (II) 2-morpholinoethan-1-amine, NMP, 110 °C, 2 hrs, (b) stacked ¹H NMR spectra of compounds **26–28**.

Each successive ring closing step has greater thermodynamic barrier due to the successively greater strain placed on the system. Whilst the first step can be carried out using the appropriate amine at room temperature, the next step requires temperatures of around 110 °C whilst the final ring closing step to form a fully planar compound requires temperatures of around 180–200 °C. In order to access the final azide-functionalised compounds

we decided to first synthesise the ring-closed hydroxy analogue before carrying out tosylation and substitution. Therefore, compound **27** was synthesised via reaction of **26** with ethanoloamine at room temperature in NMP. The ^1H NMR of **27** shows splitting of peaks *a* and *b* as their chemical equivalence is lost, as well as the formation of two triplets corresponding to the ethoxy side-chain. The morpholino-functionalised side chain was added next via an analogous procedure at 110 °C to reach compound **28**. In this case, 3 distinct triplets can be seen in the ^1H NMR (two overlapping) due to the three different environments of the original proton *a*. Five distinct doublets can also be seen for the *b* proton, with the protons directly adjacent to the remaining methoxy groups overlapping to give a total of six, corresponding to the nine aromatic protons.

Next, a ring closing reaction was carried out with **27**. The reactant was heated up to 200 °C in pyridine·HCl for 1.5 hours, after which it was cooled, the pH adjusted to 14 and extracted with DCM to give **29**. Although originally similar ADOTA trianguleniums had been purified via recrystallisation, various chromatography solvent conditions were tested and eventually a suitable combination of 50:50 DCM:MeCN with 10 mM NaPF₆ was identified. The ^1H NMR shows the expected 5 distinct proton environments. Tosylation to give **30** was subsequently carried out in DCM with tosyl anhydride and triethylamine; the corresponding set of two doublets can be seen in ^1H spectra at 6.8 and 7.1 ppm and an expected shift in the set of protons adjacent to the hydroxy/tosylate group can be seen. Finally, substitution to the azide functionalised triangulenium **31** was carried out via reaction in DMSO with sodium azide to give the product in near quantitative yield.

An analogous procedure was then carried out for the DAOTA derivative. Initially, identical conditions were used for the ring closing step (200 °C, 1.5 hours); however, NMR and mass spectrometry analysis showed that under these conditions elimination of the hydroxy group could occur to give the alkene. If left long enough this product could then react with the pyridine·HCl used as a solvent to form a pyridinium-functionalised side-chain. Pleasingly, this side-product pathway could be avoided by lowering the temperature to 170 °C and carrying out the reaction for 2 hours to give **34**. Tosylation was carried out as before,



Scheme 3.3 Synthesis of azide functionalised ADOTA and DAOTA derivatives and the corresponding stacked ¹H NMR spectra. Identical conditions used for the both synthetic pathways: (I) tosyl anhydride, NEt₃, DMF, 2 hrs, (II) sodium azide, DMSO, 24 hrs. All NMRs in MeCN-d₃ apart from **33** in DMSO-d₆ due to solubility issues.

using tosyl anhydride and triethylamine. **33** was isolated as the tosylate salt due to it precipitating out of solution, as shown by an additional set of two doublets in the ^1H NMR at 7.15 ppm and 7.4 ppm. Finally, reaction with sodium azide gave the desired product **34** again in near quantitative yield.

With the two Pt^{II} -salphens and two azide functionalised trianguleniums in hand, synthesis of the tethered conjugates was attempted. Coupling was achieved via use of the CuAAC reaction using $[\text{Cu}(\text{MeCN})_4] \cdot \text{PF}_6$ and a slight excess of the triangulenium compared with the Pt^{II} -salphen. The reaction proceeded smoothly. As the triangulenium-based probes were soluble in DCM but the final products were not, purification was carried out using precipitation. However, a small impurity was observed in the ^1H NMR spectrum for **35** and **36**, perhaps due to incomplete conversion from **13** to **25** as the addition of more of the azide did not cause it to disappear. However, in conjunction with HPLC data, the probes were deemed pure enough to continue. The ^1H spectra of compounds **35** and **36** is shown in Figure 3.3; the peaks corresponding to the Pt^{II} -salphen and ADOTA triangulenium can be clearly identified, along with a new peak corresponding to the triazole. Unsurprisingly, the imine peaks show a greater separation compared with the starting material in both **35** and **36** due to the off-centre amide linker and conjugation of the much larger triangulenium. Additionally, **36** shows upfield shifts across all peaks, indicating that protons in both the triangulenium and Pt^{II} -salphen moieties are shielded, potentially due to the increased linker length allowing for more effective $\pi - \pi$ stacking between components. Very similar trends are also seen for compounds **37** and **38** with the DAOTA appended.

Next, the absorption spectra of the compounds was measured and compared with their respective components. In all cases, the peaks of **35–38** can be assigned to Pt^{II} -salphen, the triangulenium, or a combination of them both. Although the ultimate aim of the probes was to use fluorescence and phosphorescence lifetime to separate the channel of each probe, it was pleasing to see that it would be possible to selectively excite each fluorophore using a non- or near non-overlapping wavelength (for example Pt^{II} -salphen excitation at 375 nm, ADOTA excitation at 550 nm and DAOTA excitation at 560 nm).

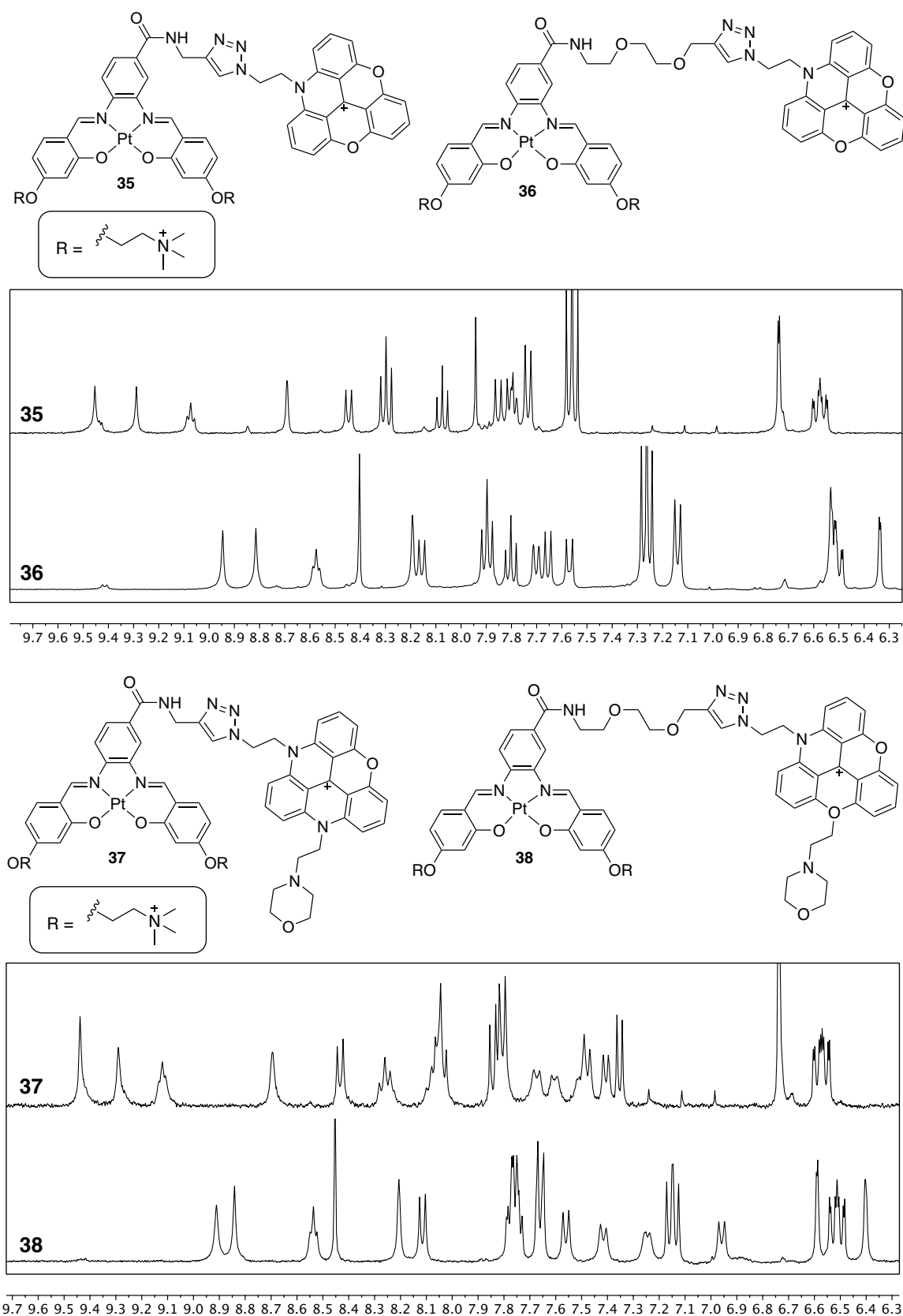


Figure 3.3 Structures and ^1H NMR spectra of compounds 35–38 (DMSO- d_6). Peaks corresponding to the Pt^{II}-salphen are shown in red whilst peaks corresponding to the ADOTA/DAOTA are shown in blue. Synthesis conditions: $[\text{Cu}(\text{MeCN})_4]\cdot\text{PF}_6$, MeCN, 16 hrs.

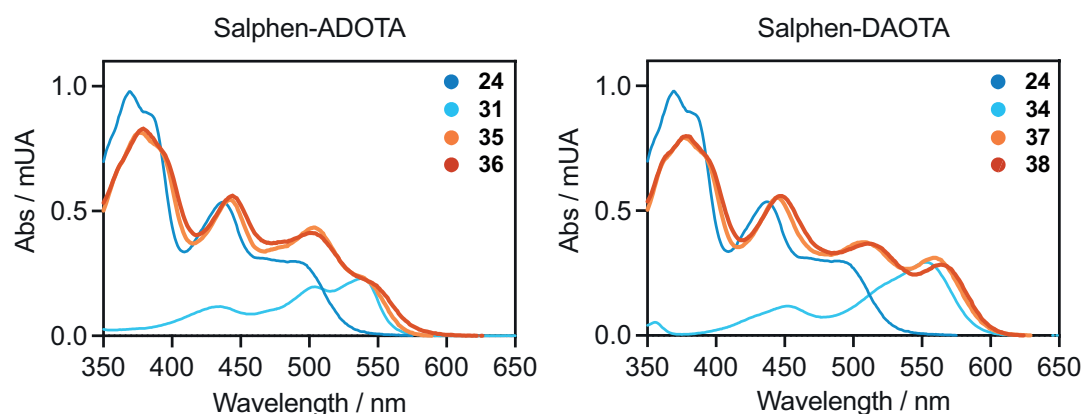


Figure 3.4 Absorbance of compounds **35–38** compared with their respective components (20 μ M in water).

3.3 DNA binding

DNA binding was subsequently carried out with probes **35–38**. Firstly, DNA titrations were carried out with G1, G2T1 and G2T6 in both Na^+ and K^+ buffers as well as CT-DNA. In each case, the probes were excited at two wavelengths, one corresponding to the Pt^{II} -salphen and one corresponding to the ADOTA or DAOTA fluorophores. A selection of the raw data plots is shown in Figure 3.5 and 3.6. The Pt^{II} -salphen channel was accessed via excitation at 375 nm and emission at 590 nm, the ADOTA channel was excited at 535 and emission measured at 575, and the DAOTA channel excited at 560 and emission measured at 600 nm. Figure 3.5a shows compound **35** with G1 and G2T1 G4 DNA in both K^+ and Na^+ buffer. Each graph shows the Pt^{II} -salphen channel in red and the ADOTA channel in blue. In all cases, the Pt^{II} -salphen showed the expected switch on effects. In contrast, large differences were seen in the emission of the ADOTA moiety. Firstly, it is important to note that the Pt^{II} -salphen is nearly completely quenched in aqueous media, in line with the previously discussed compounds whilst the ADOTA moiety shows a much higher baseline fluorescence. Upon the addition of G1(K^+) DNA a slight quenching of emission can be seen, indicating that the ADOTA is directly interacting with DNA.^[187] The addition of G2T1(K^+) led to an initial decrease in emission, followed by a slow increase as DNA concentration was increased. Switching to Na^+ stabilised buffer led to little change in the shape of the titration curves for the Pt^{II} -salphen channel, although the switch on effect was increased

(discussed subsequently). However, the addition of G1(Na⁺) led to an increase in emission for the ADOTA channel. An initial decrease followed by a slow increase in emission was observed for G2T1(Na⁺). Figure 3.5b shows the same four conditions for compound **36**, which showed a similar trends for the Pt^{II}-salphen and ADOTA channels. Overall, whilst the 'switch-on' or 'switch-off' behaviour of the ADOTA is dependent on DNA sequence and topology, the magnitude and the way in which sometimes both an increase and decrease in signal is seen make it hard to determine any unique set of signals for any one topology. This behaviour was also observed for G2T6(K⁺), G2T6(Na⁺) and CT-DNA (data in Appendix).

Figure 3.6a and 3.6b show the same set of data for **37** and **38**. The Pt^{II}-salphen channel showed the expected 'switch-on' effect across all DNAs, albeit with different magnitudes and apparent binding constants. Interestingly, the DAOTA channel displayed the exact same emission patterns as the Pt^{II}-salphen channel, although the initial emission was slightly higher as the starting compound showed incomplete quenching. This indicates that perhaps there is a single binding event for both the monomeric and dimeric G4 DNA and that neither unit behaves independently. An alternative explanation that was considered is that there is some sort of FRET effect between the Pt^{II}-salphen and DAOTA due to the overlap between the Pt^{II}-salphen emission and the DAOTA absorbance. However, this does not fully explain these results; it has been shown that DAOTA-M2 and similar derivatives display around a 2-4 fold increase in emission when bound to DNA, whereas here a much larger switch on effect is seen even when only the DAOTA channel is excited.

Next, the Pt^{II}-salphen emission was used to calculate the overall 'switch-on' (I/I_0) effect and K_a values for compounds **35–38**. This data is summarised in Figure 3.7. Firstly, **35** and **36** displayed a very similar response across monomeric and dimeric G4 DNA and K⁺ and Na⁺ buffers of around a 30-50 fold increase in brightness. In contrast, **37** and **38** had a consistent trend of the highest 'switch-on' effect seen for G2T1, followed by G2T6 and finally G1. The dimeric 'switch-on' was seen for G2T1(Na⁺) at around 160-fold, compared with only a 20-fold increase for G1(Na⁺). All the compounds showed a similar 'switch-on' effect in the presence of CT-DNA of around 15-30 fold. The K_a values were calculated using $(I-I_0)$.

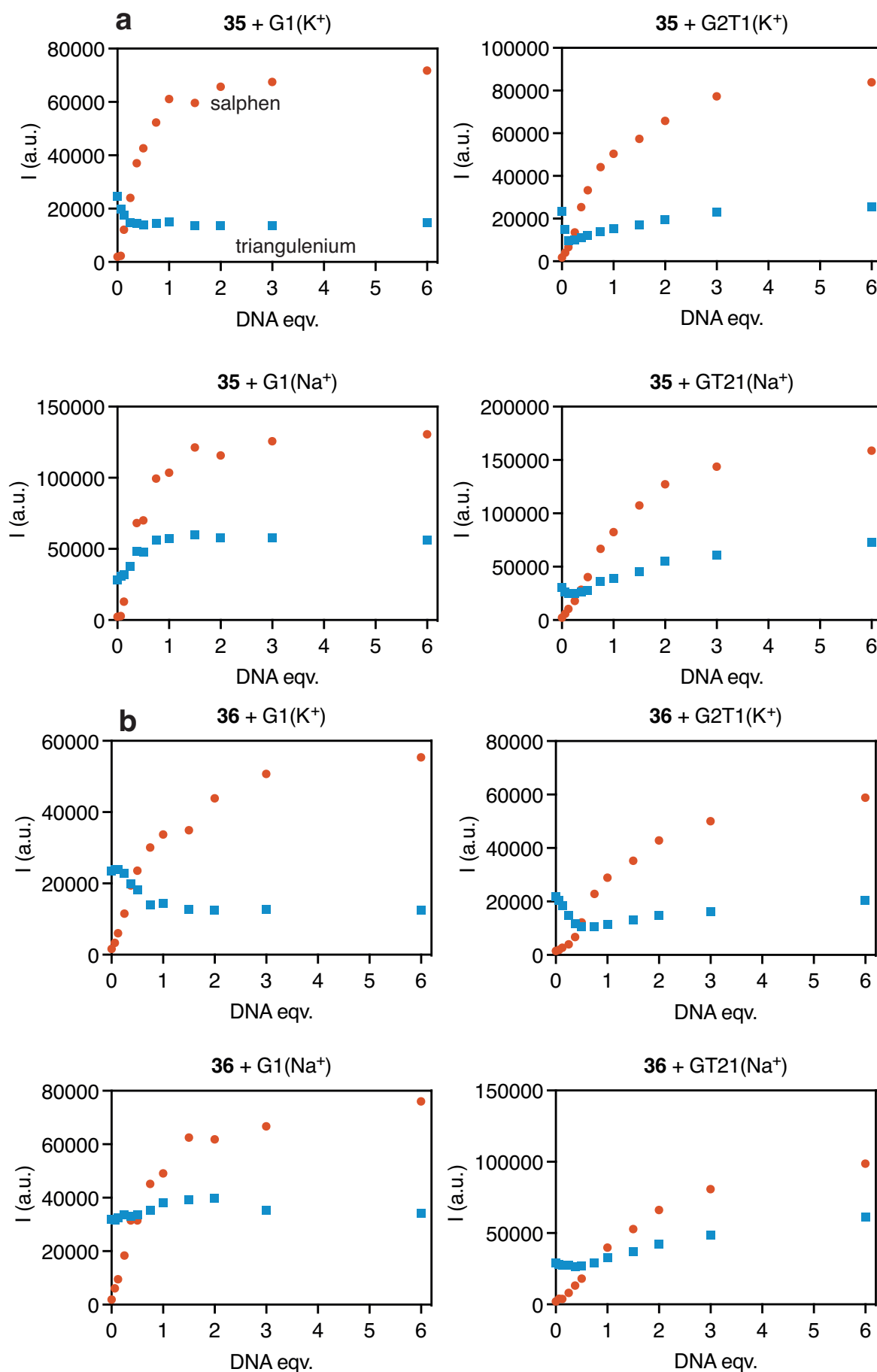


Figure 3.5 Raw data titration plots of compounds **35** (a) and **36** (b) with G1 and G2T1 G4 DNA in K⁺ and Na⁺ buffers. Pt^{II}-salphen, $\lambda_{\text{ex}} = 375$ nm, $\lambda_{\text{em}} = 590$ nm; ADOTA, $\lambda_{\text{ex}} = 535$ nm, $\lambda_{\text{em}} = 575$ nm).

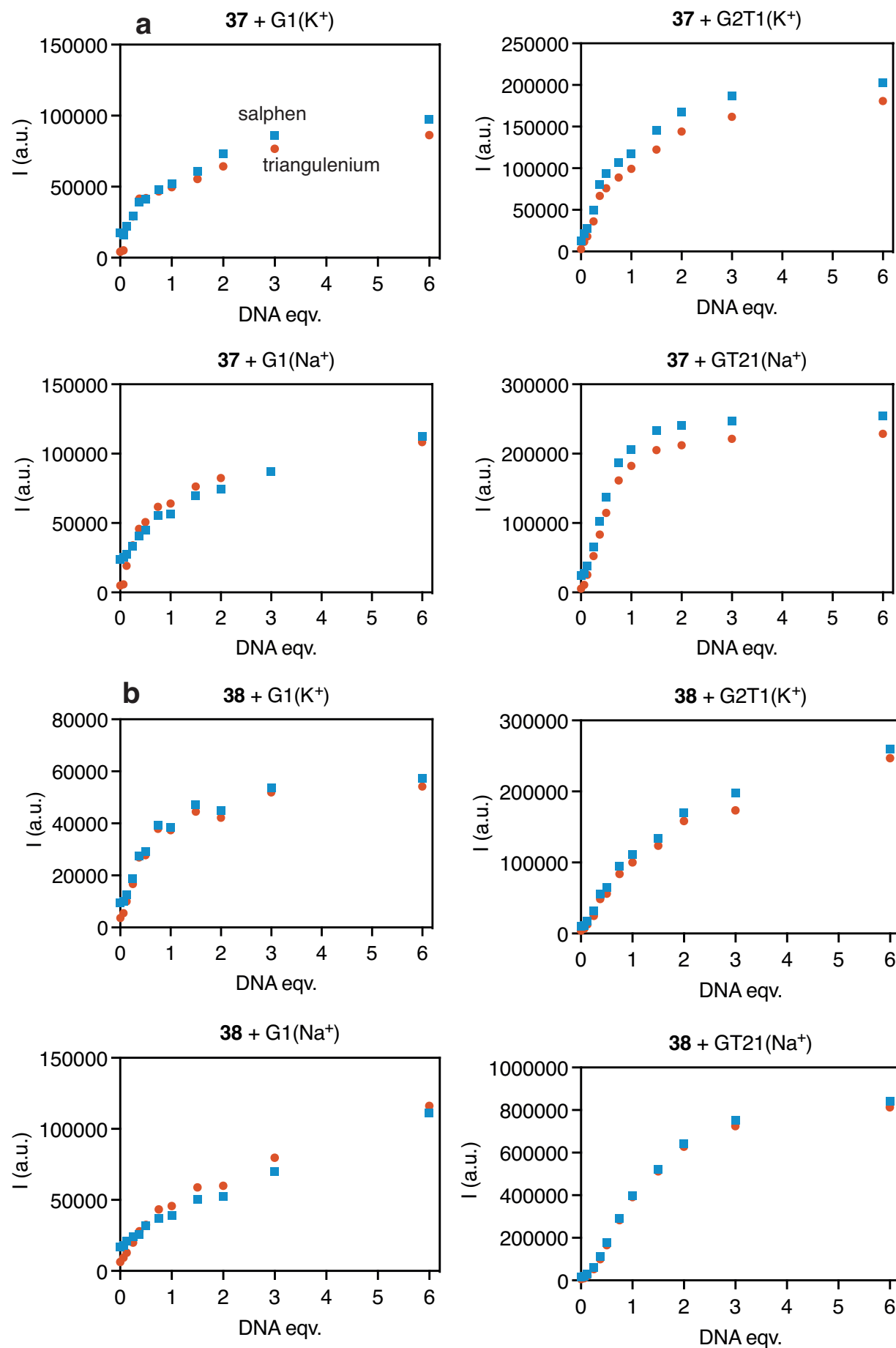


Figure 3.6 Raw data titration plots of compounds **37** (a) and **38** (b) with G1 and G2T1 G4 87 DNA in K⁺ and Na⁺ buffers. Pt^{II}-salphen, $\lambda_{\text{ex}} = 375$ nm, $\lambda_{\text{em}} = 590$ nm; ADOTA, $\lambda_{\text{ex}} = 560$ nm, $\lambda_{\text{em}} = 600$ nm).

Interestingly, the values obtained did not follow the trends seen for the intensity changes; across all four compounds the greatest binding constants were obtained for monomeric G4 DNA, both in Na⁺ and K⁺ buffers. Additionally, compound **38**, which had shown the greatest 'switch-on' effect had the lowest K_a values. Very little selectivity was seen for G4 over duplex DNA. The differences in this data are perhaps due to the multiple interactions that take place upon binding of the probe to DNA; between the Pt^{II}-salphen and DNA, between the ADOTA/DAOTA and DNA, and between the Pt^{II}-salphen and ADOTA/DAOTA. Without a much more detailed investigation into the photo-physical properties of the compounds it is hard to draw definitive conclusions.

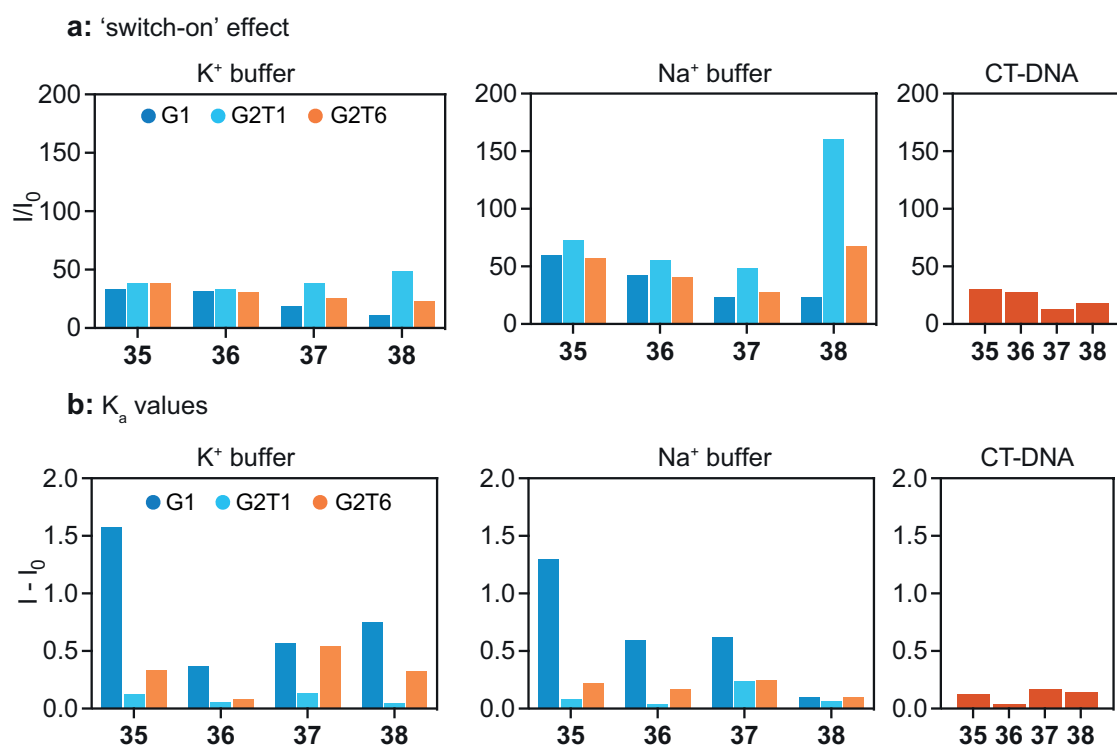


Figure 3.7 Summary of the 'switch-on' and K_a values obtained for compounds **35–38** using the Pt^{II}-salphen excitation and emission channel from the previous experiment.

It was decided that CD melting experiments could be a suitable alternative method with which to analyse compound-DNA interactions as only changes in the DNA structure are tracked. Compounds **37** and **38** with the DAOTA moiety were selected for further testing as the 'switch-on' effects indicated that these compounds could potentially display selectivity for dimeric G4 DNA. K⁺ stabilised HTelo G4 DNA has been shown to undergo a conforma-

tion change upon melting and was therefore also excluded from the CD melting experiment. Therefore, **37** and **38** were tested against Na⁺ stabilised G1, G2T1 and G2T6 G4 DNA (Figure 3.8).

As discussed previously, the monomeric Pt^{II}-salphen can stabilise both monomeric and dimeric G4 DNA, with a slight selectivity for dimeric structures (15 °C compared with 20 °C). In contrast, both **37** and **38** selectively stabilised G2T1 compared with G1 and G2T6 structures. Compound **38** in particular showed very little stabilisation of monomeric G4 DNA (0.6 °C) whilst retaining moderate dimeric G4 DNA stabilisation (9.2 °C).

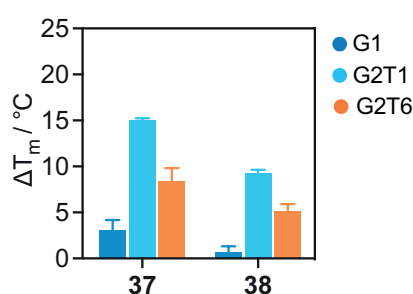


Figure 3.8 CD melting experiments with **37** and **38**. [G4 unit] = 6 μM, [ligand] = 12 μM.

Finally, some preliminary phosphorescence and fluorescence lifetime experiments were conducted with compound **38** using time correlated single-photon counting (TCSPC). These results were compared with the lifetimes of the individual components, i.e. unmodified Pt^{II}-salphen **24** and azide-functionalised DAOTA **34**. G1(Na⁺), G2T1(Na⁺) and CT-DNA were used. A 2 μM stock solution of compound was measured on its own or mixed with either 10 equivalents of monomeric G4 DNA, 5 equivalents of dimeric G4 DNA, or 100 BPE of CT-DNA. For phosphorescence lifetime, excitation was carried out at 361nm and time-resolved emission was measured at 590 nm using a 495 nm filter over time periods 340 μs and 2700 μs for compound **24** and **38** respectively. For fluorescence decay measurements, a similar procedure was carried out with the following exceptions: excitation was undertaken at 440 nm, solutions were prepared with compound **34** in place of compound **25** and decay was recorded over a 100 ns time period.

First, the fluorescence lifetimes of **34** and **38** were compared. Unfortunately, the data could not be fitted accurately and so lifetimes could not be extracted. However, from the

decays themselves a number of conclusions can be drawn. Interestingly, the azide functionalised triangulenium appeared to display much longer lifetimes when bound to multimeric G4 DNA than to either duplex or G1 DNA and the potential for **34** to distinguish between duplex, G4 and multimeric G4 structures is being explored outside the scope of this thesis. In contrast, little difference was seen for **38** between the free compound and all three DNA sequences.

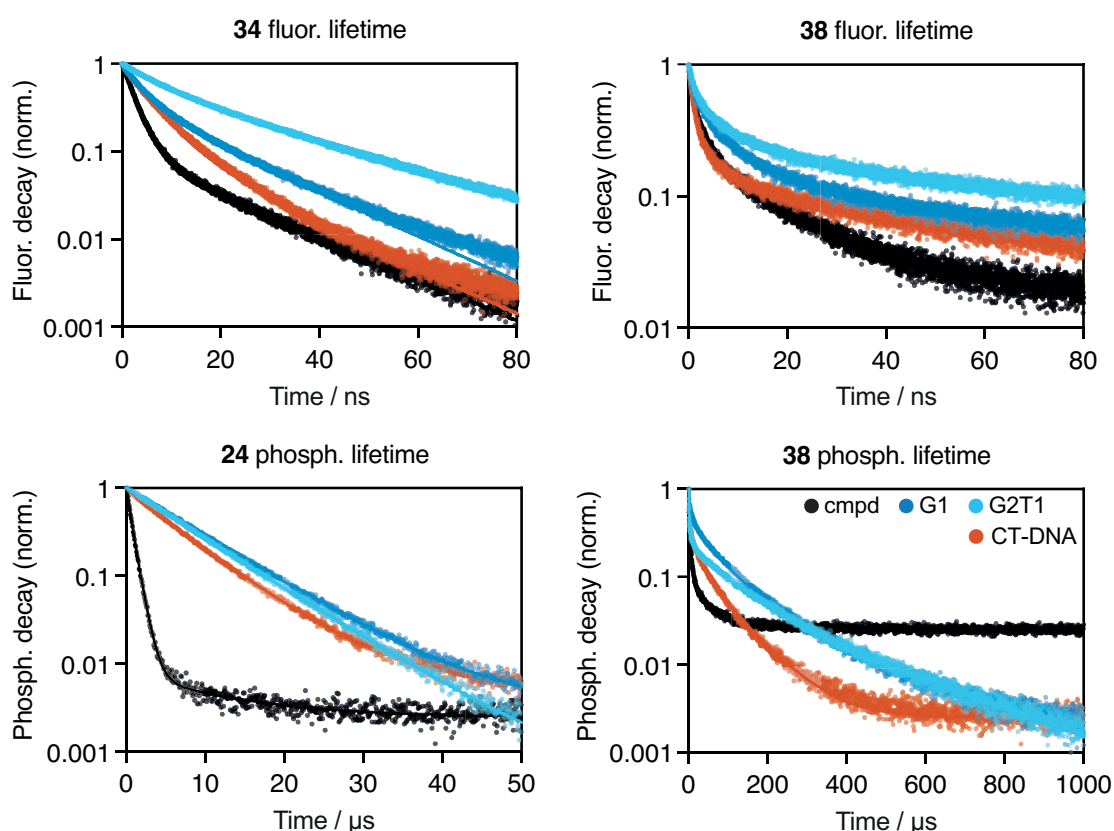


Figure 3.9 Phosphorescence and fluorescence lifetimes of compound **38** compared with the individual components Pt^{II} -salphen **24** and azide-triangulenium **34**.

Next lifetimes were measured on a the phosphorescence timescale previously obtained for Pt^{II} -salphens (1-10 μs). Figure 3.9a shows the lifetimes of the free compound (black) with the three previously mentioned DNA sequences. In all cases, the curves could be fitted to a mono-exponential decay. As expected, the lifetime of the probes significantly increased with the addition of DNA (from 0.7 μs to 6-8 μs). However, little discrimination was seen between duplex, monomeric and dimeric G4 DNA. Intriguingly, very different results were seen for compound **38**; all the lifetimes (even the free complex) were of an order of mag-

nitude higher and in the range of 10–120 μ s. Additionally, all topologies displayed highly distinct lifetimes (free complex, 13 μ s; CT-DNA, 41 μ s; G1, 81 μ s; G2T1, 119 μ s). It is important to note here that there was an initial fast decay on the fluorescence timescale. This was then used to calculate what percentage of the overall emission corresponded to the μ s-range decay. These very long lifetimes led us to consider by what mechanism this emission takes place as there was no plausible reason for a direct 10-fold increase in phosphorescence lifetime. One alternative is that of triplet–triplet energy transfer. In this case, a triplet energy sensitizer is used to generate an excited triplet state (T_1) in the donor molecule. Indeed, in many cases transition metal complexes (including Pt^{II} -salphens) are used due to their near unity ISC caused by the heavy atom effect.^[188–190] The transition from the excited state back to the ground (S_0) state is coupled via a non-radiative transition with the excitation of the acceptor from its ground state (S_0) to an excited (T_1) state, meaning it is spin-allowed. This leads to a long lived triplet state centred on the acceptor ligand. Interestingly, the lifetime of this triplet state has been shown to be around 80–100 μ s,^[188,189] and furthermore the donor and acceptor units do not have to be part of the same aromatic system and can even be brought together in space via host–guest interactions.^[191] Although a much more detailed photo-physical investigation is needed, these preliminary results might indicate a very interesting way of distinguishing between DNA topologies; the interaction of compound **38** with DNA clearly has a significant effect on the long component of the emission lifetime which could be due to the relative positions of the TTET donor and acceptor units.

3.4 Conclusions and future work

In conclusion, a new set of Pt^{II} -salphen–triangulenium conjugate compounds have been synthesised and tested for their ability to selectively target multimeric G4 DNA structures. Some preliminary experiments have been carried out which show that the probes have potential to discriminate between duplex, monomeric, and dimeric G4 DNA by way of emission lifetime. However, further analysis of the photo-physical phenomena causing the sig-

nificantly lengthened lifetimes is required. One option of doing so would be to synthesise an analogue with a non-emissive Ni^{II}-salphen tethered to the triangulenium. This would allow for us to study how the triangulenium portion of the molecule interacts with DNA without any potential energy transfer effects. In addition, a more detailed investigation into the potential TTET processes taking place is needed. One way of doing so would be to use transient absorption spectroscopy to determine whether the triplet state of the triangulenium does indeed become occupied.

Chapter 4

Rotaxanes as cages for DNA-targeting ligands

4.1 Background

A currently under-explored area of G4-related research is the development of probes in which G4 interaction can be controlled through an external trigger. As summarised in Section 1.5.1, only a small number of probes have been synthesised which fulfil the criteria for a switchable or triggerable G4 ligand. We were therefore interested in further developing this approach. However, we were also interested in the broader application of these types of strategies to drug delivery.

One major problem in drug development is that the efficacy of many drugs can be limited by undesirable properties such as poor aqueous solubility, low bioavailability, and “off-target” interactions in the body.^[192–194] To combat these deficiencies, and enhance the pharmacological profile of therapeutic agents, various approaches have been taken. For example, drugs can be encapsulated in various carriers from which it undergoes controlled release. This can lead to a number of advantages such as reduced and less frequent dosing and lowered off target effects.^[195] Another interesting method is through the synthesis of prodrugs in which covalent attachment of various protecting groups can be used to improve bioavailability or block a drug’s mechanism of action.^[196,197] This again allows either for controlled release via for example enzyme cleavage of esters or in the case of photoprotecting groups (PPG), a highly specific light triggered activation.^[198]

An alternative method of caging ligands is via the formation of interlocked molecules, which combine aspects of encapsulation and covalent prodrug formation. Mechanically interlocked molecules consist of at least two components connected via a mechanical bond: they are not covalently linked to one another but also cannot be separated with-

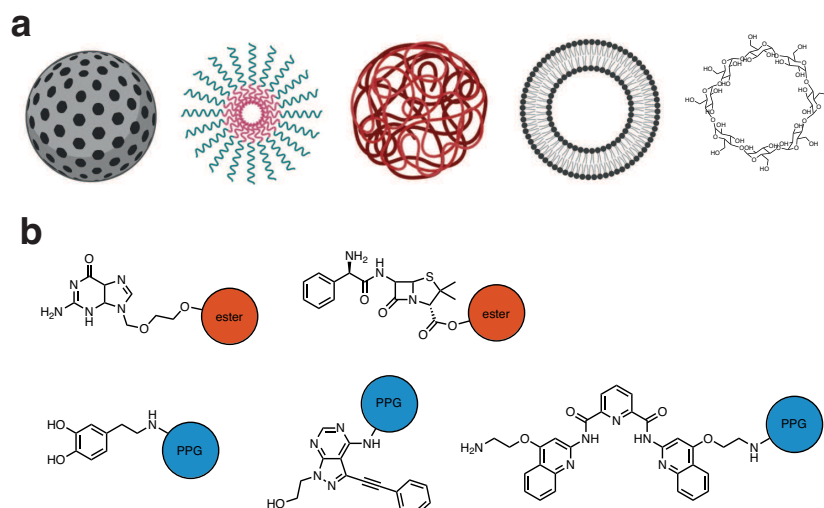


Figure 4.1 Controlled drug release. (a) encapsulation methods including nanoparticles, micelles, polymers and cyclodextrins. (b) covalent attachment of protecting groups to form prodrugs. Both autonomous (for example via enzymes) and triggered (via photoactivation of photo-protecting groups [PPG]) methods of release can be achieved.

out breaking a covalent bond.^[199] The two most common examples are [n]catenanes and [n]rotaxanes, where n is the number of components. Catenanes consist of two or more interlocked macrocycles, whereas rotaxanes comprise of an axle component threaded through a macrocycle. Bulky stopper groups prevent the macrocycle from dethreading. The continuing development of novel methods to synthesise interlocked structures has meant that it is now possible to reach highly complex molecular assemblies in relatively few synthetic steps and from simple starting materials.^[200–208] This has included a range of mechanically bound molecules capable of exploiting intramolecular motion for molecular switches and machines, catalysis, as well as the incorporation of sensing motifs which can report binding via redox or photochemical mechanisms.^[209–212] However, their utilisation in biological applications has remained under-explored.^[213] The few existing examples all utilise rotaxane based structures. This has included polyrotaxanes, in which macrocycle drug conjugates are threaded onto an axle and undergo slow controlled release over time.^[214–217] Other examples have employed rotaxanes as triggerable nano-valves to form mechanised silica nanoparticles which contain a desired drug to be released.^[218]

A feature of interlocked architectures is that each component shields each other due to being locked closely in space. This characteristic has been employed in relatively few bi-

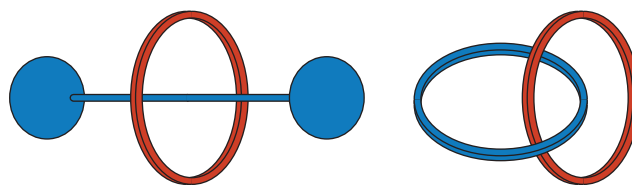


Figure 4.2 The archetypal classes of mechanically interlocked molecules: rotaxanes (left) and catenanes (right).

ological systems. For example, the macrocycle can be used to shield reactive or unstable fluorophores and peptides in order to successfully deliver them into cells.^[219–223] Other examples have included a self cleavable programmable drug delivery system, in which a self immolating macrocycle can be triggered to open by certain enzymes, releasing a drug.^[224] More recent examples include a metallohelicate caged by inclusion in a rotaxane with a cucurbituril macrocycle^[225] and the inhibition of duplex formation of an unnatural DNA strand by the circling of a biocompatible 1,2,3-triazole linker by a macrocycle, suppressing its ability to act as a primer for PCR amplification.^[226] However, these approaches have had limitations; Leigh and co-workers drug delivery rotaxane was a highly complex structure and also only examined autonomous delivery, whilst Hannon and co-workers' metallohelicate rotaxane primarily focused on how binding might be inhibited.

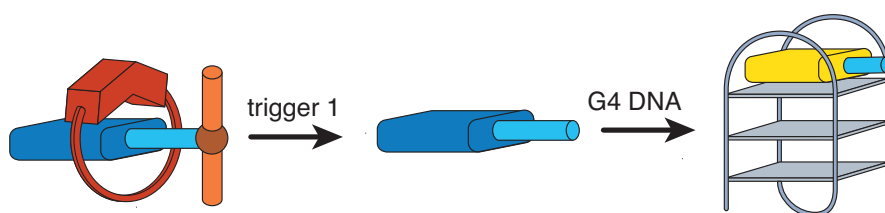


Figure 4.3 Schematic showing the activation mechanism in which G4 binding is initially blocked via the presence of a macrocycle and then subsequently activated allowing for G4 DNA stabilisation.

We therefore set out to address some of these concerns and develop a new platform for drug delivery via rotaxane formation. In particular, we were interested in how these systems could be applied to G4 DNA targeting ligands, as we imagined that the presence of macrocycle sitting orthogonally to the G4 binding site would effectively block any DNA interaction. Then, via inclusion of a triggerable stopper group we could liberate the G4

binder, switching binding back on.

The subsequent Chapter is split into the following parts: (1) synthesis and characterisation of a new family of interlocked molecules; (2) interaction of the rotaxanes with DNA; (3) investigation into activation and (4) application of the rotaxane based drug delivery system towards cancer cells.

4.2 Synthesis

4.2.1 Probe design

The majority of synthetic methods used to construct interlocked molecules rely on some form of templating effect. This has included both anion and cation templated mechanisms. Of particular importance has been the development of active metal template (AMT) synthesis (primarily the copper catalysed azide alkyne cycloaddition, or CuAAC, reaction).^[227–229] This method utilises a macrocycle with a copper binding site and an axle component split into two stoppers functionalised with an alkyne and azide respectively. Through the use of a sub-stoichiometric amount of copper, a click reaction can be forced to occur through opposite faces of the macrocycle, forming the triazole and mechanical bond. The metal therefore plays an 'active' role, templating the molecule and catalysing the mechanical bond-forming reaction. Pioneered by Leigh and co-workers, the use of the CuAAC reaction has played a pivotal role in the development of mechanically interlocked systems due to mild reaction conditions, high yields, and tolerance for a wide array of solvents and reaction conditions.

In order to build our interlocked drug delivery system from relatively simple building blocks, we chose a previously established macrocycle containing a bipyridine Cu^I binding site cyclised with a PEG chain, which has been used to form a variety of rotaxanes in excellent yield.^[230] With an alkyne functionalised Pt^{II}-salphen already in hand (synthetic details in Chapter 2), the remaining component to design was an azide functionalised cleavable stopper group. We aimed for a modular design approach, in which the mechanism of activation could be easily modified. Additionally, in order to conserve the binding capabil-

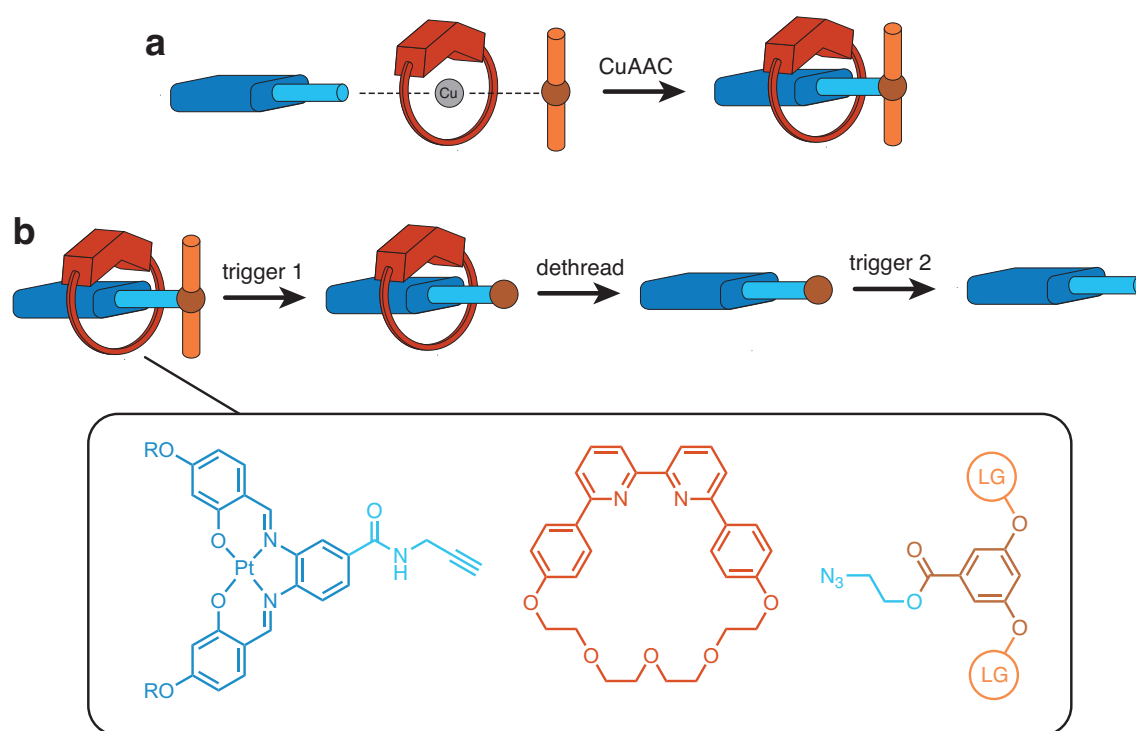


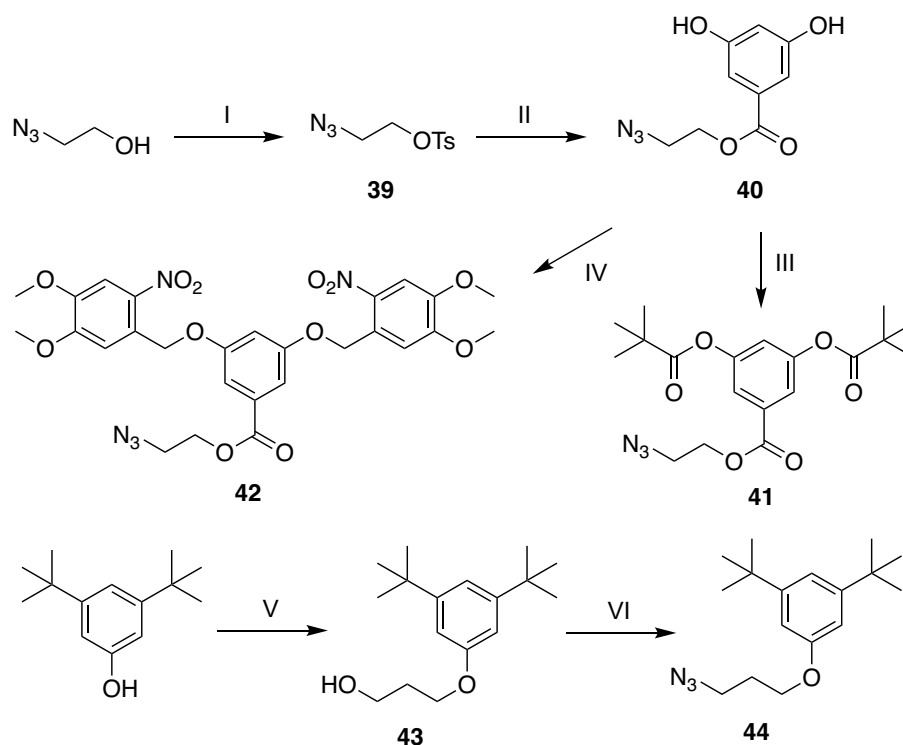
Figure 4.4 Probe design. (a) Active metal template synthesis; a macrocycle with a binding site templates a click reaction through opposite faces of the macrocycle and (b) diagram showing two stage sequential activation mechanism.

ities of the Pt^{II}-salphen, we wanted to ensure that the majority of the steric bulk of the second stopper was removed upon activation. To achieve both these considerations the azide stopper was designed around a 3,5-dihydroxybenzoic acid core, in which an azide functionalised linker was attached via an ester bond to the carboxylic acid and the two remaining hydroxy groups could be used to attach cleavable leaving groups. We hoped that functionalising both the 3 and 5 positions would lead to a stopper group large enough to block macrocycle de-threading. The release of the Pt^{II}-salphen from the interlocked assembly would therefore occur via the following steps: (1) a primary activation trigger, removing the two cleavable leaving groups and (2) de-threading of the macrocycle, exposing the linker connecting the Pt^{II}-salphen and remaining 3,5-dihydroxybenzoate fragment. This would allow for a third step: (3) the cleavage of the ester bond by endogenous esterases, leaving a hydroxy-functionalised Pt^{II}-salphen. Importantly, the presence of the macrocycle and the two cleavable stopper groups in the initial interlocked assembly would inhibit ac-

cess to the ester bond and allow for sequential triggering of the primary (cleavable leaving groups) and secondary (esterase) activation mechanisms.

4.2.2 Rotaxane synthesis and characterisation

The azide functionalised 3,5-dihydroxybenzoate core was synthesised via a two-step reaction in which 2-azidoethanol was reacted to form the tosylate (**39**) before being added to 3,5-dihydroxybenzoic acid to form **40**. From this compound it would be possible to add numerous cleavable protecting groups; however, we chose to focus on two orthogonal methods of activation. Firstly, in order to study an autonomous delivery method, we functionalised the stopper with two pivalic esters chosen for their steric bulk (compound **41**). Any subsequent rotaxanes formed with this stopper would undergo two sequential enzymatic ester cleavages. Whilst having an esterase triggered primary activation mechanism does not provide any selectivity (compared to, enzymes which are up-regulated in cancer cells for example), they are well established in prodrug formation and easy to study.

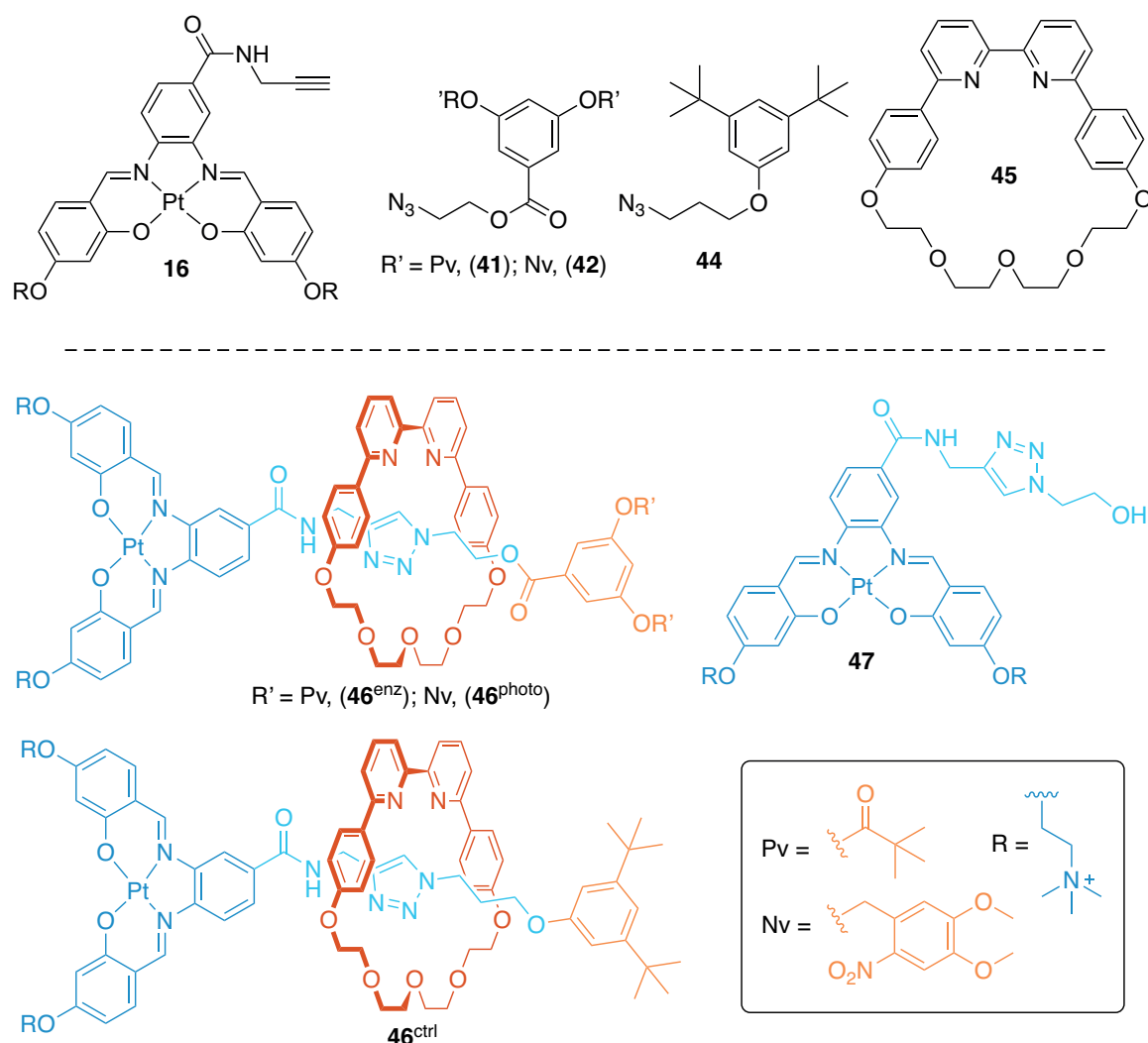


Scheme 4.1 Synthesis of rotaxane stoppers: (I) TsCl, NEt_3 , DCM, (II) 3,5-dihydroxybenzoic acid, K_2CO_3 , DMF, (III) pivalic acid, EDC·HCl, DMAP, DCM, (IV) nitroveratryl bromide, K_2CO_3 , DMF, (V) 3-bromopropanol, K_2CO_3 , DMF, (VI) TsCl, NEt_3 , followed by NaN_3 .

Photo-activation was another mechanism we were particularly keen to utilise. Rather than autonomous release, in which rotaxane break-down and Pt^{II}-salphen release would start immediately, a light activated primary trigger would allow for a much higher degree of spatiotemporal control as the interlocked assembly would remain intact until irradiation. The increased control afforded by such systems has led to a dramatic rise in the use of photo-protecting groups for pharmacological purposes. To synthesise a light-activated rotaxane we used a nitroveratyl photo-protecting group as the cleavable stoppers, which can be activated via irradiation with 365 nm light. Compound **42** was formed via reaction of **40** with nitroveratyl bromide. Finally, a stopper with no cleavable components (compound **44**) was synthesised from the literature via a two-step reaction. This particular stopper was chosen in order to approximately match the linker length separating the azide from the aromatic core in compounds **41** and **42** (*synthesised by Dr James Lewis*).

The complete set of synthesised rotaxane components and rotaxanes is shown in Scheme 1.2. The two cleavable rotaxanes, denoted **46**^{enz} for the enzymatically activated compound and **46**^{photo} for the photo-activated compound, along with the non-cleavable control rotaxane (**46**^{ctrl}) were prepared via AMT synthesis. First, one equivalent of macrocycle **45** was dissolved in acetonitrile and combined with a slightly sub-stoichiometric quantity of copper(I) tetrakis acetonitrile in order to ensure that the click reaction primarily took place through the macrocycle. Next one equivalent of Pt^{II}-salphen **15** and the respective stopper component were added and the reaction was stirred overnight. Next, the solvent was removed and the residue re-dissolved in DCM. Interestingly, whilst Pt^{II}-salphen **15** was only sparingly soluble in DCM, all the rotaxanes showed good DCM solubility. This is presumably due to the appendage of lower polarity groups along with reduced $\pi - \pi$ stacking interactions between the Pt^{II}-salphen aromatic surfaces. The DCM solutions were then washed with an aqueous solution of NH₄OH with 100 mM EDTA in order to remove the copper from the rotaxane cavity. Finally, the rotaxanes were purified via preparative TLC.

Pt^{II}-salphen **47**, the compound designed to be released after the primary and secondary activation mechanisms, was also synthesised in order to confirm that the compound could



Scheme 4.2 Synthesis of rotaxanes and active Pt^{II}-salphen complex: a general rotaxane synthesis procedure was followed in which 1 eqv. **45** was combined with 0.96 eqv. [Cu(CH₃CN)₄] $\cdot$ PF₆ followed by the addition of 1 eqv. of **15** and the appropriate stopper. Pt^{II}-salphen **47** was synthesised via click reaction with [Cu(CH₃CN)₄] $\cdot$ PF₆ and azidoethanol.

still bind to G4 DNA and to provide a positive control for successful rotaxane activation. This compound was simply prepared via click reaction of **15** with 2-azidoethanol.

The rotaxanes were next characterised via NMR spectroscopy. Peaks were assigned using a combination of ¹H and 2D NMR. Figure 4.5 shows rotaxane **46^{enz}** compared with the clicked Pt^{II}-salphen **47** (shown in blue) and macrocycle **47** (shown in red) as an example. Peaks corresponding to the cleavable stopper component are shown in orange.

Compared with the non-interlocked components, large shifts can be seen for the chemical environments of the protons in the interlocked assembly. Upfield shifts characteristic

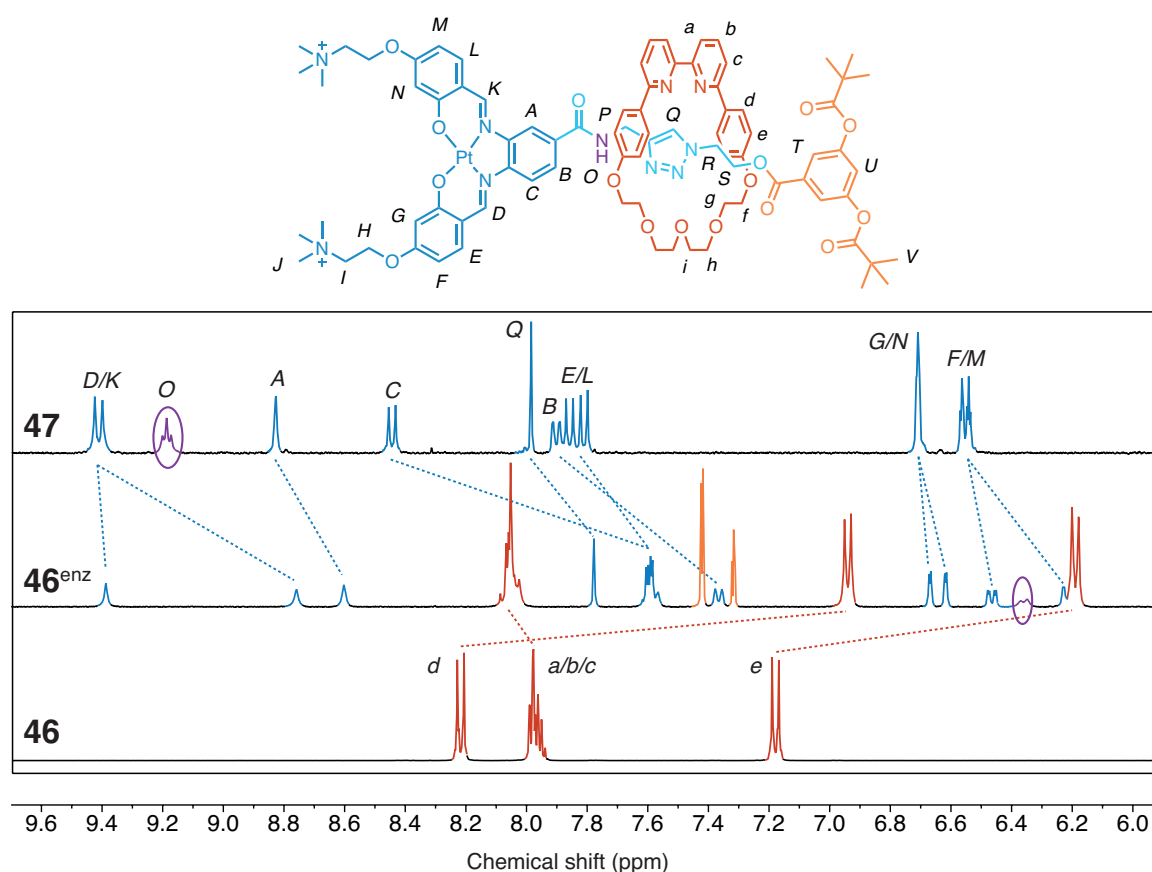


Figure 4.5 Rotaxane ^1H NMR characterisation. Section of the ^1H NMR (DMSO- d_6 , 400 MHz) spectrum of Pt^{II} -salphen complex **47** (blue), rotaxane **46^{enz}**, macrocycle **47** and axle component (orange).

of rotaxane formation can be seen as each component shields the other; for example, the macrocycle peaks *d* and *e* shift around 1.2 ppm upfield, from 8.2 to 7.0 and 7.2 to 6.0 ppm respectively. There is comparatively little change for protons *a/b/c* presumably as they point directly outwards so are unlikely to be effected by the presence of the axle component. Upfield shifts are also seen for many of the Pt^{II} -salphen protons. This effect is much more pronounced for one side of the salphen than the other due to the off-centre placement of amide linker. For example, whilst imine proton *D* stays at around 9.4 ppm, imine proton *K* shifts from 9.4 to 8.7 ppm. Interestingly, the singlet at 8.8 ppm corresponding to proton *A* shifts by less (0.2 ppm) than the doublets corresponding to protons *C* and *B* (8.4 to 7.6 and 7.8 to 7.3 ppm respectively). This is perhaps due to the orientation of the macrocycle around the axle leading to specific interactions of these protons (*A/B/C*) with either the aromatic or PEG linker side of the macrocycle. The largest shift in chemical environment

can be seen for the amide bond proton (*O*). Upon rotaxane formation an upfield shift can be observed (9.2 to 6.3 ppm), indicating that the macrocycle sits directly over the amide bond and that the proton itself is involved in hydrogen bonding to the bipyridine motif in the macrocycle. This is further evidenced by a broadening of the peak. The other potential resting site of the macrocycle is the triazole. However, a comparatively smaller upfield shift is seen for proton *Q* (7.9 to 7.8 ppm). Taken together with the upfield shifts observed for the Pt^{II}-salphen core, the ¹H proton data indicates that the macrocycle sits directly adjacent to and partially over the Pt^{II}-salphen, positioning it ideally to block any potential DNA interactions. A comparison of the ¹H spectra of **46**^{enz}, **46**^{photo} and **46**^{ctrl} shows that the chemical shifts are generally conserved and therefore that the macrocycle resting site is over the amide bond in all cases (Figure 4.6).

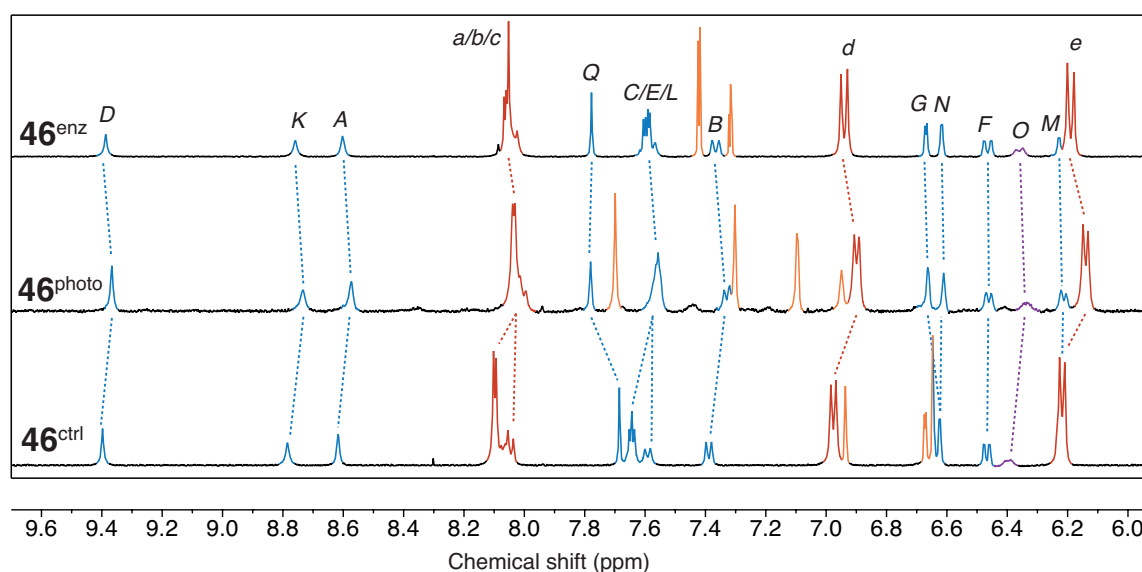


Figure 4.6 Stacked ¹H NMR spectra of rotaxanes **46**^{enz}, **46**^{photo} and **46**^{ctrl}.

Next, ROESY NMR was used to confirm the interlocked nature of the rotaxanes and that the macrocycle was likely positioned over the Pt^{II}-salphen core. ROESY NMR uses the nuclear Overhauser effect to identify protons which undergo cross relaxation and are therefore near one another in space. As there are no through-bond limitations, protons on separate components which are locked together should display cross peaks. Figure 4.7 shows the ROESY NMR for **46**^{enz} in which extensive cross coupling is seen between the macrocycle protons *a-e* and Pt^{II}-salphen protons *D*, *K*, *A* and *B*. Similar spectra for **46**^{photo}

and **46**^{ctrl} can be found in the Supporting Information.

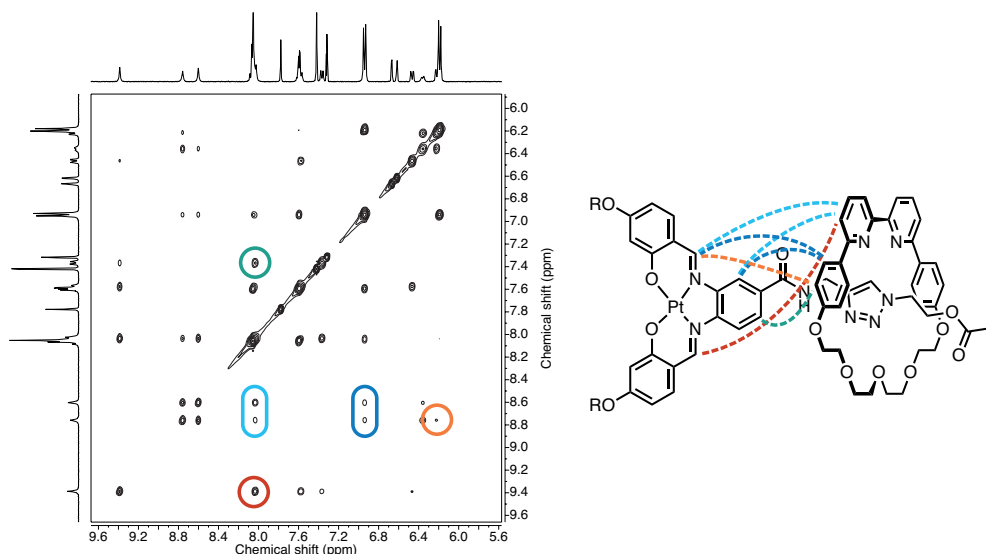


Figure 4.7 2D ROESY NMR of rotaxane **46**^{enz} with a highlighted selection of cross-peaks between protons.

Finally, the rotaxane structures were investigated using Density Functional Theory (DFT). The energy of the **46**^{enz} was minimised as a representative example using a B3LYP functional with 6-31G and LANL2DZ basis sets and water as the solvent. As can be seen from Figure 4.8, the structure matched our hypothesis well, with the macrocycle positioned off-centre and directly adjacent to the Pt^{II}-salphen core, with a bifurcated hydrogen bond between the amide bond proton and the bipyridine with bond lengths of 2.4 and 2.5 Å. From the space filled models it is evident that this macrocycle placement leads to a significant blocking of the Pt^{II}-salphen core.

Before progressing to biophysical and cellular assays, the photophysical properties of the rotaxanes were studied and compared with the free Pt^{II}-salphen **47**. Minimal differences were observed in the absorbance spectra in the region corresponding solely to the Pt^{II}-salphen core (Figure 4.9a, 400-550 nm). Contrastingly, the emission spectra of the four compounds varied significantly. As discussed in the previous Chapter, Pt^{II}-salphens are emissive with a characteristic primary peak at around 590 nm and a second peak at approximately 625 nm. However, in aqueous media, free Pt^{II}-salphens such as **47** undergo aggregation induced quenching. This effect can be seen in Figure 4.9b with the emission of

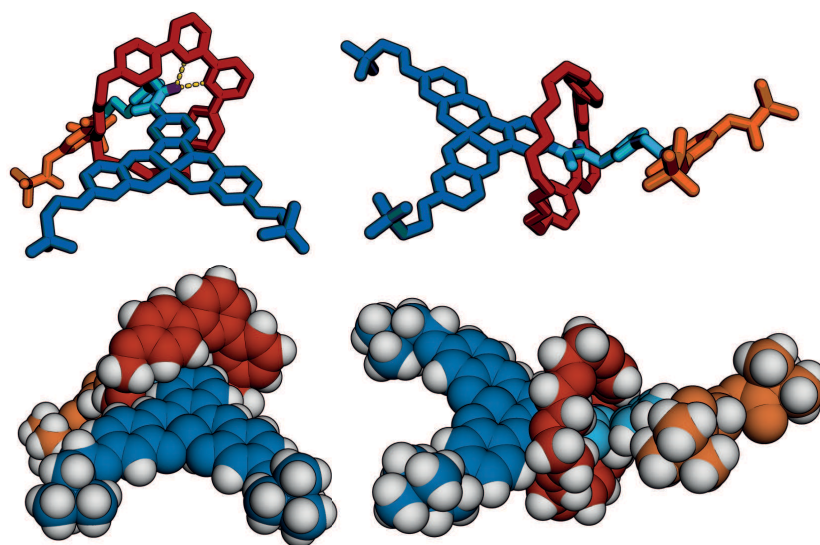


Figure 4.8 Energy minimised structure of rotaxane **46^{enz}** alongside a space-filled model. Strong hydrogen bonds (2.4 and 2.5 Å) can be seen between the amide proton on the axle and the bipyridine nitrogen and are highlighted in yellow.

47 shown in red. In comparison, rotaxanes **46^{enz}**, **46^{photo}** and **46^{ctrl}** all display varying degrees of enhanced emission. This is presumably due to a reduction in aggregation induced quenching caused by the presence of the macrocycle. Interestingly, the emission for **46^{enz}** is around four-fold higher than for **46^{photo}** and **46^{ctrl}**. This data would suggest that the differing size and hydrophobicity of the secondary stopper group can effect the amount and type of aggregation in aqueous media.

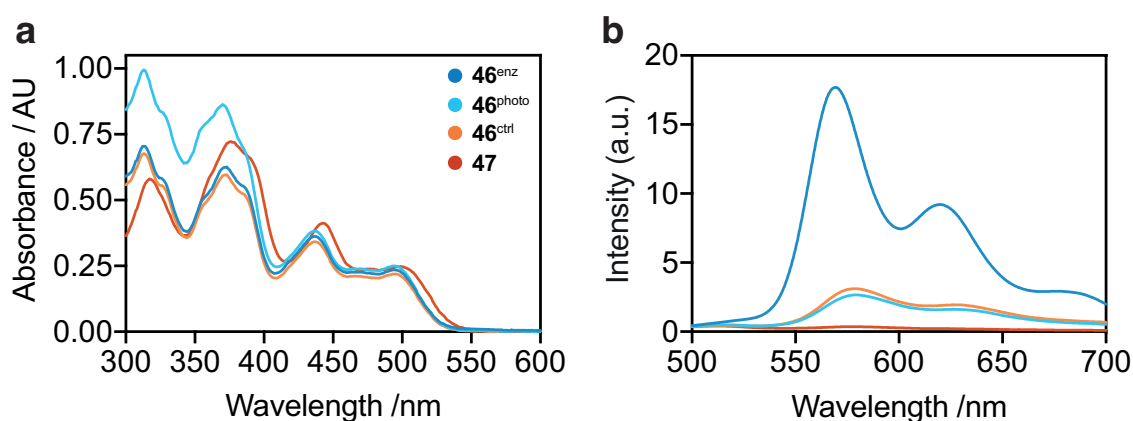


Figure 4.9 Absorbance (a) and emission (b) spectra of rotaxanes **46^{enz}**, **46^{photo}**, **46^{ctrl}** and Pt^{II}-salphen **47** (all at 50 μ M in water), showing similar absorptivity but significantly different emission intensities ($\lambda_{\text{ex}} = 440$ nm)

4.3 DNA binding

The DNA binding properties of the rotaxanes were compared to the free Pt^{II}-salphen **47**. A range of G4 DNA topologies were tested alongside duplex DNA. The G4 structures tested were HTelo (Na⁺) (parallel), HTelo (K⁺) (hybrid) and *c-Myc* (antiparallel). In all cases, the rotaxanes showed virtually no switch on effect whilst the free Pt^{II}-salphen **47** showed excellent stabilisation of all G4 structures, with K_a values in the 10^6 M⁻¹ range and a slight preference for the HTelo (Na⁺) over HTelo (K⁺) and *c-Myc* ($K_a = 1.1 \pm 0.2 \times 10^6$ M⁻¹ compared to $7.0 \pm 0.1 \times 10^5$ and $6.8 \pm 0.1 \times 10^5$ M⁻¹, respectively). Additionally, the complex displayed around 500-fold selectivity for G4 over duplex DNA ($K_a = 1.8 \pm 0.1 \times 10^3$ M⁻¹). Affinity constant for the rotaxanes could not be obtained as the fluorescence increase was negligible. It is important to note that from the whilst the photo-physical characterisation showed that the rotaxanes had higher baseline fluorescence than the free Pt^{II}-salphen, the values

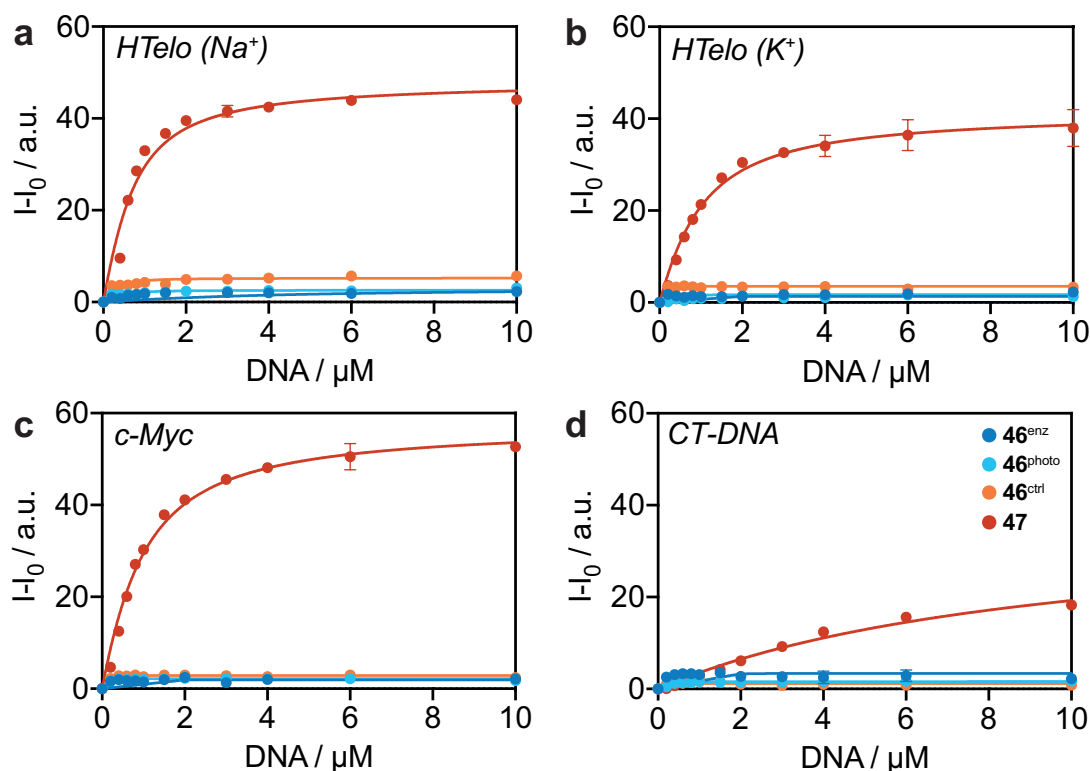


Figure 4.10 DNA titrations with rotaxanes 46^{enz} , 46^{photo} , 46^{ctrl} and Pt^{II}-salphen **47** (all at 2 μM) with HTelo (Na⁺), HTelo (K⁺), *c-Myc* and CT-DNA respectively. For G4 forming sequences, concentration is given per strand whilst for CT-DNA concentration is per base pair of DNA ($\lambda_{\text{ex}} = 440$ nm, $\lambda_{\text{em}} = 590$ nm).

were not close to the emission maximum obtained during the titrations and therefore if the rotaxanes could bind to DNA an enhancement in emission would still be expected to be seen. This data therefore indicates that the macrocycle does indeed significantly hinder the ability of the Pt^{II}-salphen core to interact with both G4 and duplex DNA.

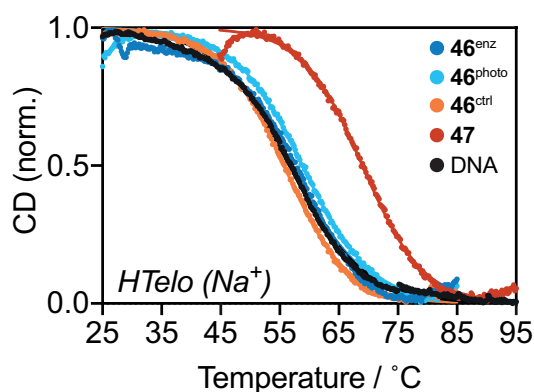


Figure 4.11 CD melting spectra of HTelo (Na⁺) (shown in black) (5 μM) and HTelo (Na⁺) with with rotaxanes 46^{enz}, 46^{photo}, 46^{ctrl} and Pt^{II}-salphen 47 (10 μM).

Next, CD melting experiments were carried out in order to confirm the DNA binding properties of the complexes. HTelo (Na⁺) was selected as the greatest binding constants had been obtained. The DNA was first melted without any complex present, before being followed by the addition of the rotaxanes and 47. As can be seen from Figure 4.11, little-to-no thermal stabilisation occurred upon addition of any of the rotaxanes, whilst the free Pt^{II}-salphen complex 47 showed good stabilisation (12.1 °C). As this assay exclusively measures changes in the properties of the DNA, it provides good corroborating evidence that the lack of switch on effect seen in the fluorescence titrations is indeed due to the lack of interaction between the rotaxanes and DNA.

4.4 Activation experiments

Activation experiments were carried out carried out and monitored using LCMS and HPLC analysis. First, the retention times of the three rotaxanes 46^{enz}, 46^{photo} and 46^{ctrl}, macrocycle 45 and the free Pt^{II}-salphen 47 were obtained. An eleven minute 5-95% gradient of MeOH in water with 0.1% TFA was used. As can be seen from Figure 4.12, good separation

was seen; the rotaxanes generally eluted at around 8 minutes, whilst the macrocycle eluted at 6.8 minutes and the free Pt^{II} -salphen at 4.1 minutes.

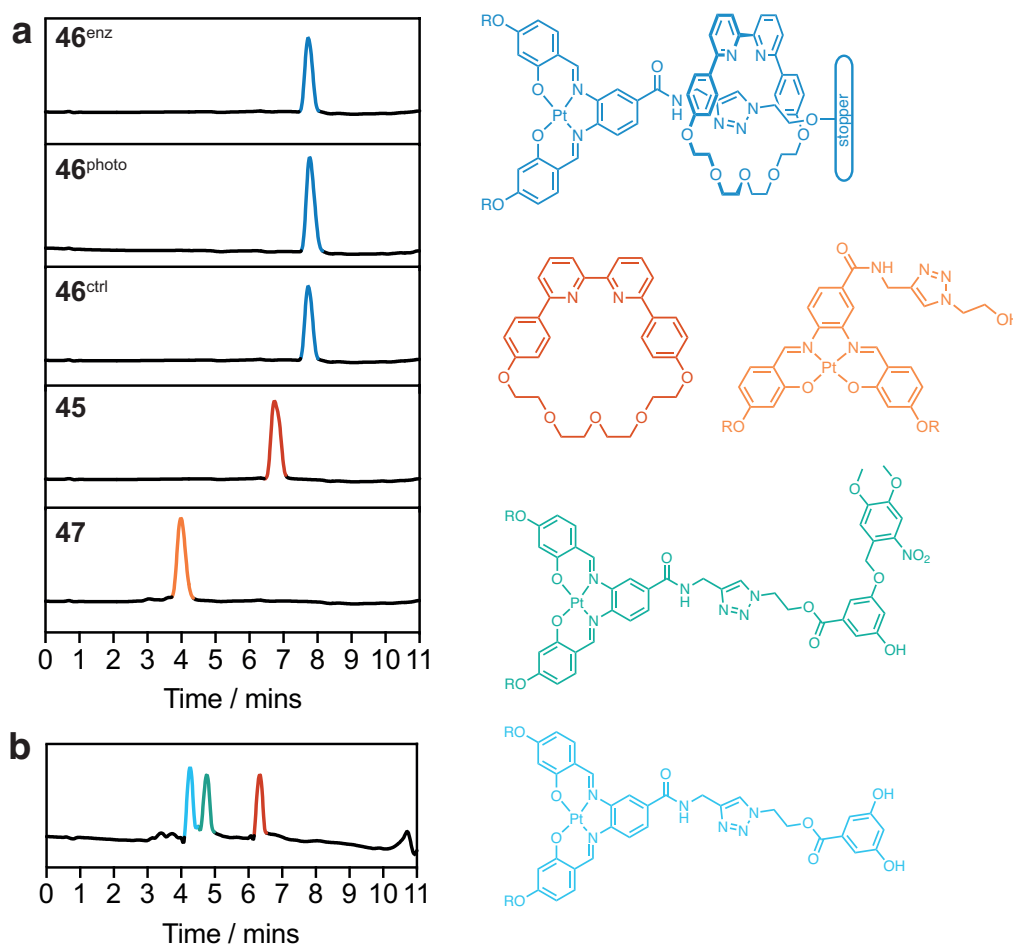


Figure 4.12 Retention times for activation experiments. (a) HPLC traces for **46^{enz}**, **46^{photo}**, **46^{ctrl}**, macrocycle **45** and Pt^{II} -salphen **47** and (b) calculation of the retention times of the intermediate complexes using a partially activated sample of **46^{photo}**.

Next, we set out to obtain the retention times of the intermediate products. This had to be done separately and not during the activation experiments due to restrictions on what solvents we could use for LCMS analysis. Additionally, we wanted to avoid unnecessary synthesis of the intermediate complexes. Therefore an experiment with **46^{photo}** was carried out in which the sample was irradiated for 20 minutes in pure water and injected for LCMS analysis. Three peaks were identified; the macrocycle peak, an intermediate complex in which the macrocycle had dethreaded and one nitrovertyl group remained (4.8 minutes), and an intermediate complex in which the macrocycle had dethreaded and both nitrovertyl groups had been cleaved (4.5 minutes). From this, we extrapolated that the same pattern of

elution times would be seen for 46^{enz} , in which a singly cleaved intermediate would elute before the double cleaved intermediate. With this data in hand, HPLC analyses of various activation mechanisms were carried out.

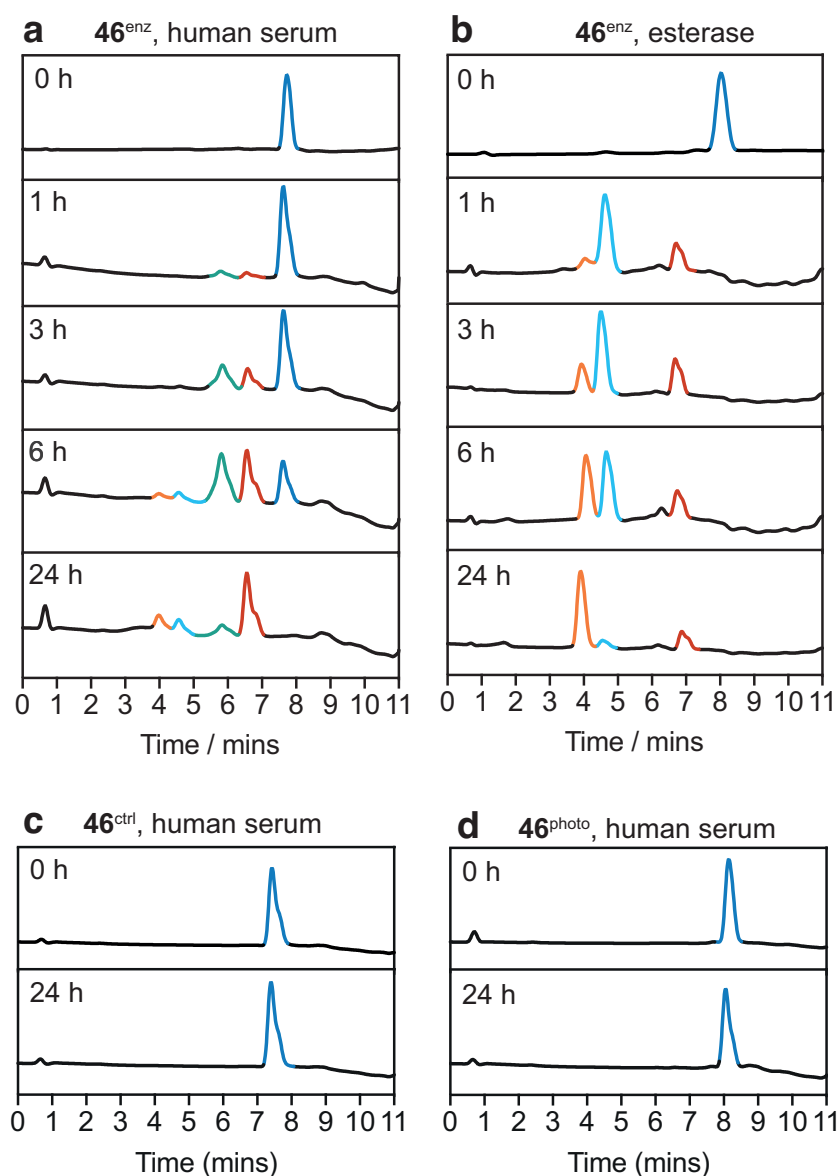


Figure 4.13 Stability and activation experiments. (a) 46^{enz} incubated in human serum at 37 °C for up to 24 hours, (b) 46^{enz} incubated with 50 equivalents of PLE per micromole at 37 °C for 24 hours and (c–d) 46^{ctrl} and 46^{photo} (absent of irradiation) incubated in human serum at 37 °C for 24 hours respectively.

First, the stability of all the rotaxane complexes in PBS buffer was confirmed; after 24 hours no complex breakdown was observed. Next, the rotaxanes were incubated in human serum for 24 hours at 37 °C. At prescribed timepoints a sample was withdrawn and dena-

tured for 5 minutes at 95 °C (an experiment in which the rotaxanes with no enzyme were heated to 95 °C for 5 minutes and analysed via HPLC confirmed they were stable under these conditions). After denaturing, the solution was centrifuged and the supernatant submitted for HPLC analysis. As human serum contains various esterases, we expected to see that under these conditions **46^{enz}** would undergo activation whilst **46^{photo}** and **46^{ctrl}** would not. Indeed, this was confirmed; after 24 hours both **46^{photo}** and **46^{ctrl}** showed no signs of cleavage whilst the HPLC trace for **46^{enz}** had no rotaxane peak and a clearly identifiable macrocycle peak. Unfortunately it was also clear that the protein precipitation step was causing the Pt^{II}-salphen intermediates to also precipitate. Therefore the experiment was repeated in PBS buffer using porcine liver esterase (PLE). In this case, rotaxane **46^{enz}** was treated with 50 equivalents per micromole and incubated at 37 °C. Rotaxane breakdown occurred much more rapidly, with full cleavage within one hour. No intermediate with a singly cleaved pivalic ester was seen and after 24 hours near complete conversion to the free Pt^{II}-salphen had occurred.

Next, the photo-activation of **46^{photo}** was tested in both buffer and pure water as the solvent. Various aliquots of **46^{photo}** were prepared, and placed 5 cm from a 365 nm wavelength UV light. At prescribed timepoints a sample was withdrawn and injected for HPLC analysis. As can be seen from Figure 4.14, in both cases full rotaxane breakdown was complete within 10 minutes. The cleavage of a single nitroveratyl group evidently led to enough of a reduction in steric bulk to allow for macrocycle dethreading, shown by the concurrent appearance of the red and teal peaks. This first step was also much faster than the cleavage of the second nitroveratyl group, perhaps due to the formation of the hydroxy product having a slightly deactivating effect. Alternatively, the rotaxane could constrain the stopper group in a more reactive conformation. After 60 minutes of irradiation in water, the majority product was the expected intermediate with both nitroveratyl groups cleaved and the secondary ester bond still intact. However, in PBS buffer further reaction was seen in which there was hydrolysis of the ester bond, leading to the formation of the free Pt^{II}-salphen complex. The reason for this further reaction seen only in buffer is unknown. The

previous activation experiments had shown that esterase was able hydrolyse the axle ester bond once the cleaved intermediate was reached; therefore no further experiments were carried out with the photo-activated rotaxane.

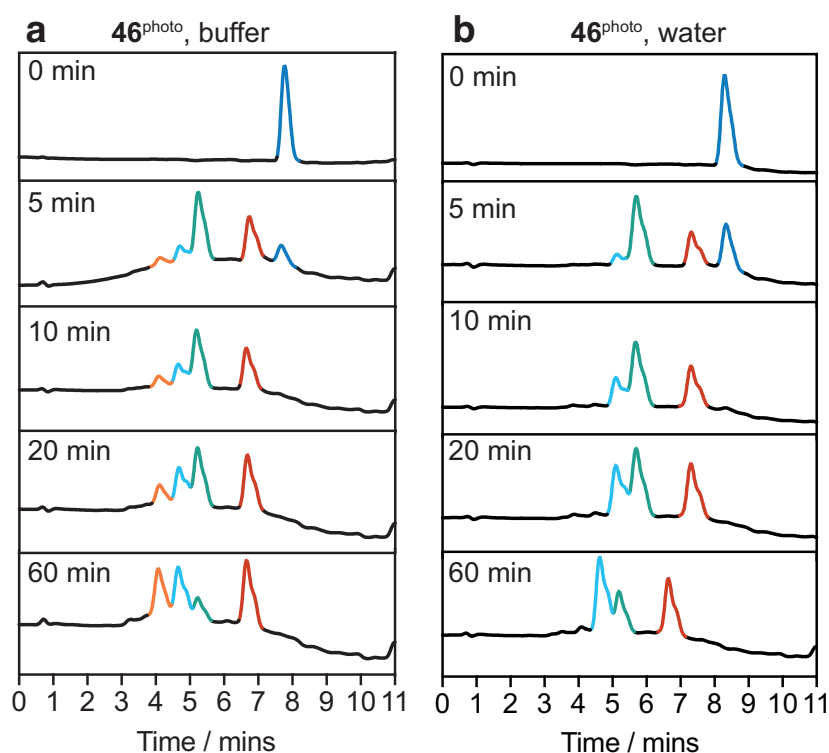


Figure 4.14 Irradiation activation of 46^{photo} using 365 nm light over 1 hour in (a) PBS buffer and (b) water.

Finally, the DNA binding properties of a free Pt^{II} -salphen generated through the above activation mechanisms was tested. A sample of 46^{enz} was incubated with 50 μM of PLE for 24 hours at 37 $^{\circ}\text{C}$, before being directly used for a series of fluorescence titrations. The sample was titrated against HTelo, c-Myc and CT-DNA alongside a negative control of 46^{enz} with no PLE and a positive control of Pt^{II} -salphen **47** with PLE. Treatment of 46^{enz} with PLE led to excellent G4 stabilisation, nearly in line with the positive control (*c-Myc*: 46^{enz} +PLE, $K_a = 4.7 \pm 0.8 \times 10^6 \text{ M}^{-1}$; 2, $K_a = 2.4 \pm 0.3 \times 10^6 \text{ M}^{-1}$; HTelo: 46^{enz} +PLE, $K_a = 1.0 \pm 0.3 \times 10^6 \text{ M}^{-1}$; 2, $K_a = 1.0 \pm 0.4 \times 10^6 \text{ M}^{-1}$). The slightly reduced total fluorescence response was presumably due to a small quantity of rotaxane not fully converted to **47**. However, the system evidently worked as desired.

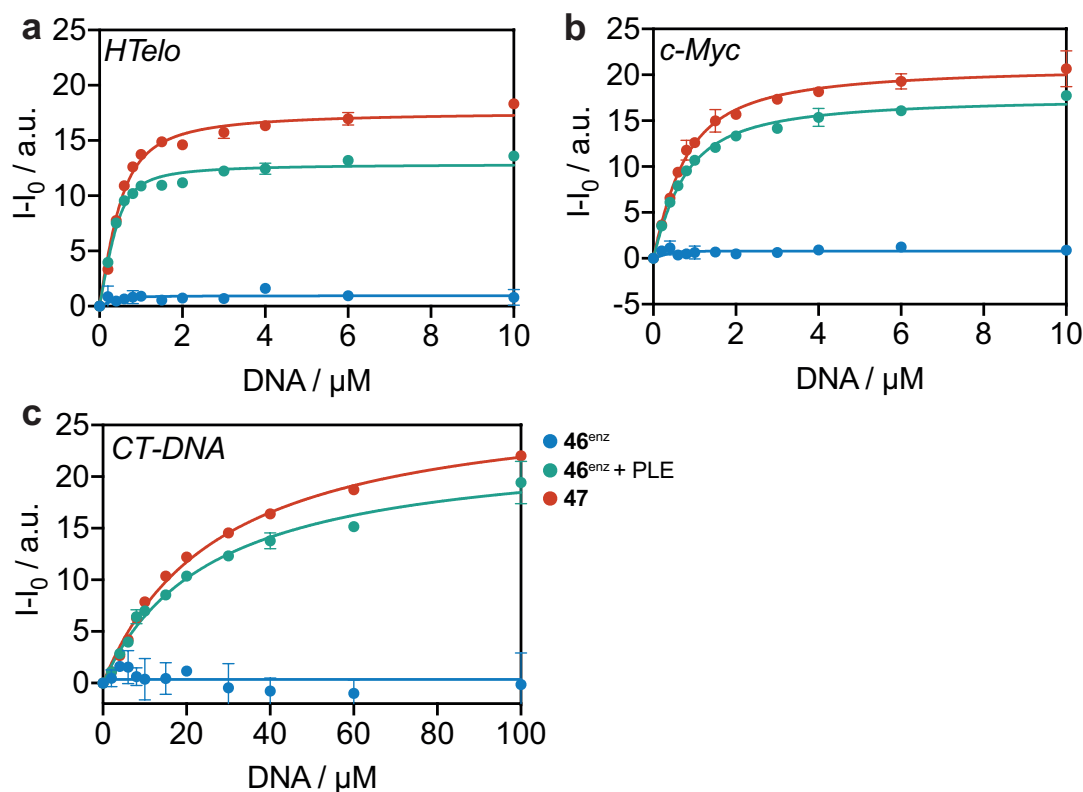


Figure 4.15 Activation experiments in which rotaxane **46^{enz}** and Pt^{II}-salphen **47** were treated with 50 equivalents of PLE per micromole and incubated at 37 °C in PBS for 24 hours, after which the solution was used for fluorescence titrations with (a) HTelo G4 DNA, (b) *c-Myc* G4 DNA and (c) CT-DNA ($\lambda_{\text{ex}} = 440 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$).

4.5 Cellular studies

Confocal microscopy in this Section was carried out by Dr Peter Summers. Having established the DNA binding properties and validated the potential for activation, we moved onto studying the cellular properties of the compounds. There were a number of key questions we wished to address: (1) Are the rotaxanes cell permeable and are there any differences compared to the free Pt^{II}-salphen? (2) If they are internalised, do they bind to DNA? (3) Can irradiation be used to trigger the release of the Pt^{II}-salphen in cells?

A combination of fluorescence confocal microscopy and cell viability assays were used to ascertain whether compounds were internalised and whether they were cytotoxic. For the cell viability assays U2OS osteosarcoma cells were seeded and grown for 24 hours, after which they were treated with varying concentrations of the appropriate compound and incubated for a further 24 hours. The viability of the cells were then measured using an

MTS/PMS assay. Next, live cell fluorescence confocal microscopy was carried out using non-cytotoxic doses of the compounds. This was firstly to determine the potential cellular localisation of the compounds, but also to confirm that any lack of cytotoxicity was not just due to poor uptake. The data is displayed side-by-side in Figure 4.16.

Surprisingly Pt^{II}-salphen **47** showed no inhibition of cell growth. As it has previously been shown that similar compounds with piperidine side chains were cytotoxic in the micromolar range, this implied that these compounds had very poor uptake, which was confirmed with the imaging experiment which showed no fluorescence response even at 20 μ M. This is perhaps due to the cationic nature of the compound as highly charged species are well-known to have difficulty crossing the cellular membrane. In contrast, all three rotaxane compounds were shown to be taken up by U2OS cells. Rotaxane **46**^{enz} inhibited cell growth in a dose-dependent manner with an IC₅₀ of 24 μ M whilst both **46**^{photo} and **46**^{ctrl} displayed little-to no inhibitory effects. In all three cases uptake was confirmed by fluorescence microscopy in which a punctuate staining pattern was observed. The dose dependent inhibition displayed by **46**^{enz} is likely due to gradual hydrolysis of the pivalic esters by endogenous enzymes and the subsequent release of the active Pt^{II}-salphen. As the uptake of **46**^{photo} and **46**^{ctrl} was confirmed, the low cytotoxicity of these compounds is presumably due to the effective caging of the Pt^{II}-salphen by the macrocycle. The complete lack of cytotoxicity seen for **46**^{photo} was particularly pleasing. The increased cellular uptake of the rotaxanes compared with the free Pt^{II}-salphen is perhaps due to the increased lipophilicity the interlocked complex and partial shielding of the cationic side-chains by the macrocycle.

Next, triggered photo-uncaging of **46**^{photo} was tested. U2OS cells were again seeded and grown for 24 hours, followed by addition of the compound. The cells were incubated for 3 hours to allow for a period of compound uptake, after which the plates were exposed to 20 minutes of 365 nm UV irradiation. The cells were then incubated for 21 hours and the cell inhibition quantified using an MTS/PMS assay. A number of control experiments were carried out; an identical procedure was followed using **46**^{ctrl}, the cytotoxicity of macrocycle

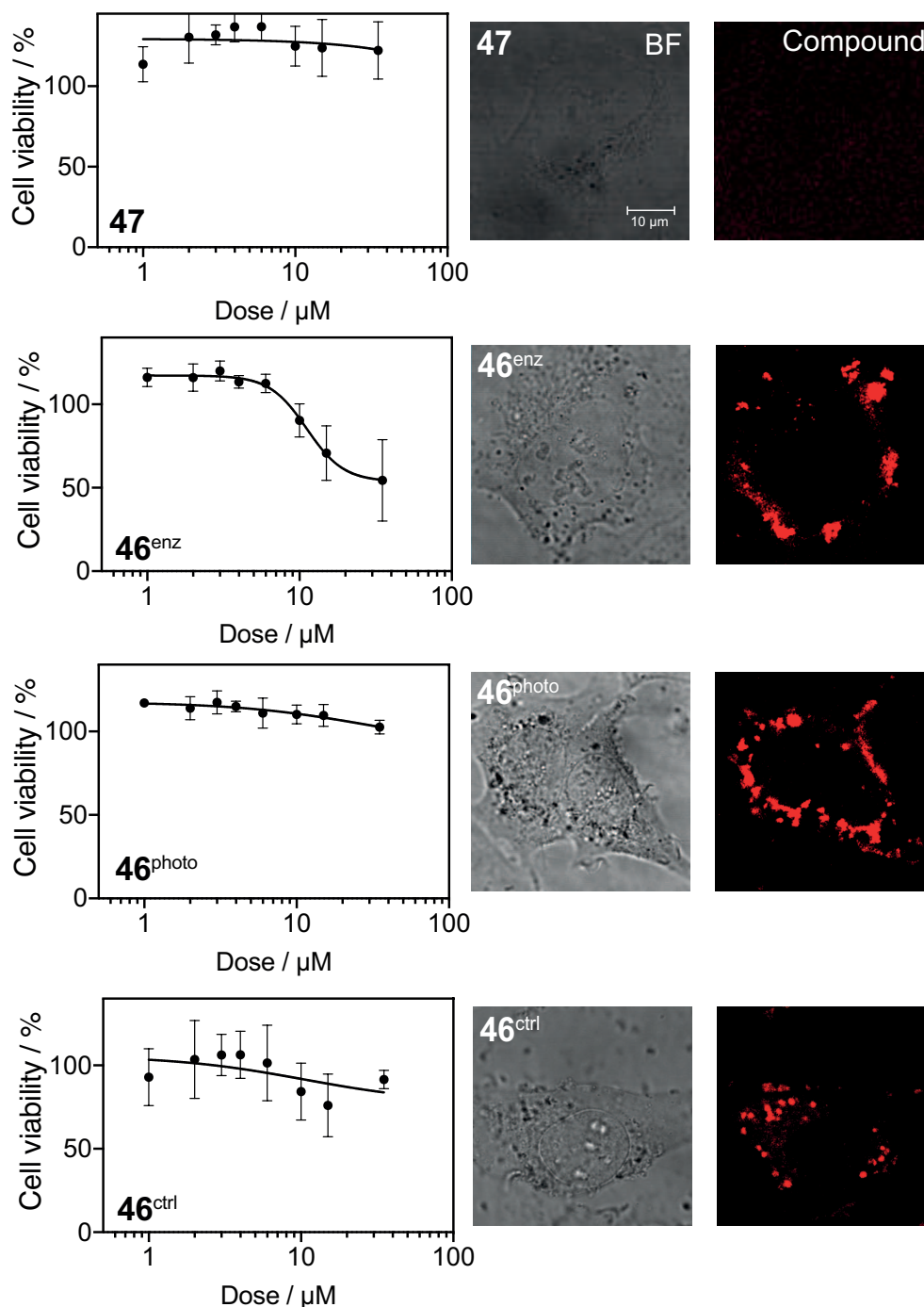


Figure 4.16 Cell viability and localisation experiments with Pt^{II} -salphen **47** and rotaxanes **46^{enz}**, **46^{photo}** and **46^{ctrl}**. Cell viability was measured at 24 hours. Live cell fluorescence imaging experiments were collected using 20 μM for Pt^{II} -salphen **47**, 2 μM for **46^{enz}** and **46^{photo}** and 10 μM for **46^{ctrl}** ($\lambda_{\text{ex}} = 458 \text{ nm}$, $\lambda_{\text{em}} = 525\text{-}700 \text{ nm}$). All images to same scale.

45 and 3,5-dihydroxybenzoic acid were measured and the effect of UV irradiation only was tested. Exposure of cells incubated with **46**^{photo} with UV irradiation led to a dramatic decrease in cell viability, with an IC₅₀ of 3.4 μ M and near complete cell death seen at 10 μ M. No corresponding change in cell viability was seen for **46**^{ctrl} and additionally, the other fragments released upon stopper cleavage were shown to be non-cytotoxic. Finally, the effect of UV irradiation only was shown to be minimal. Taken in concert, these results show that the photo-uncaging mechanism of activation is highly effective in a cellular environment and that the release of the Pt^{II}-salphen causes cell death. Additionally, it provides further

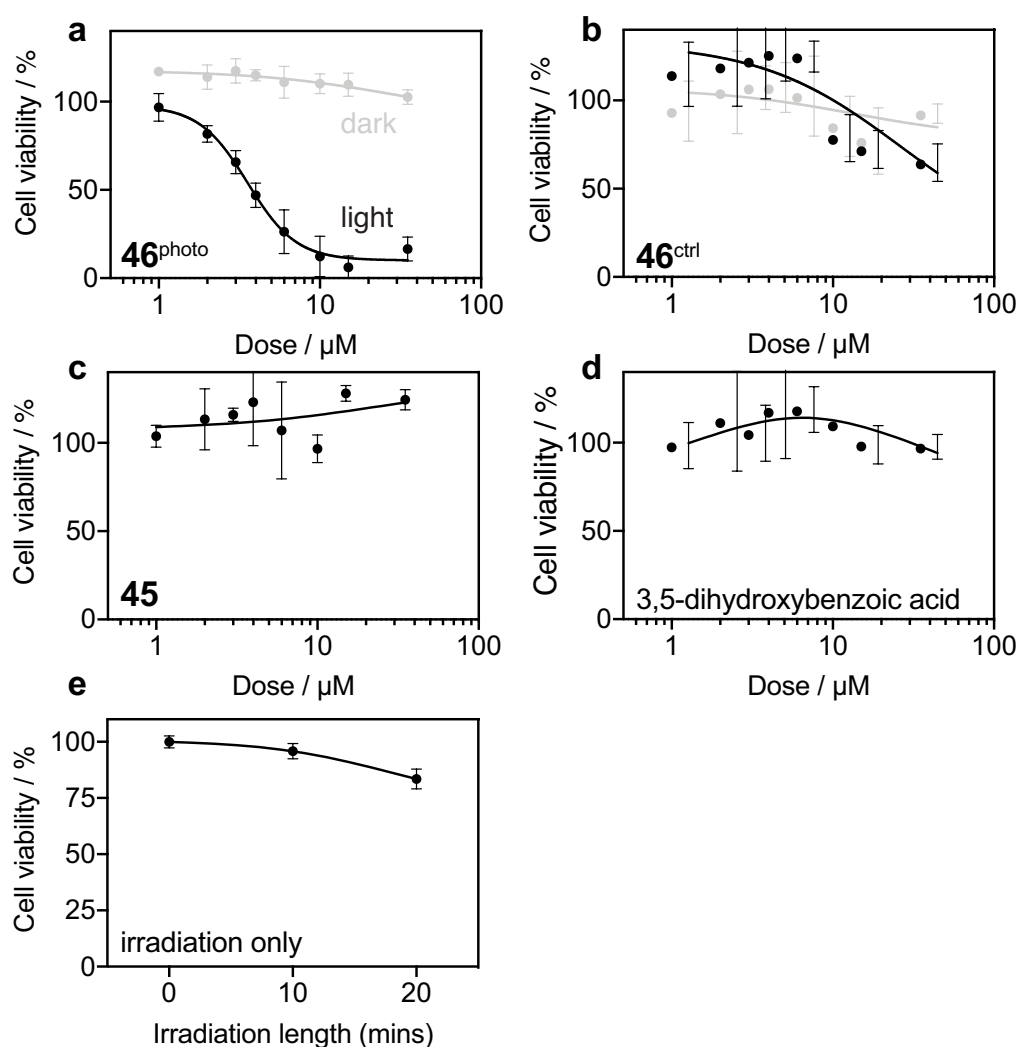


Figure 4.17 Cell viability experiments. (a,b) Cell viability of rotaxanes **46**^{photo} and **46**^{enz} respectively with 20 minutes irradiation (black) compared with no irradiation (grey), (b) cell viability of macrocycle **45**, (c) cell viability of 3,5-dihydroxybenzoic acid and (e) effect of irradiation on cell viability.

evidence that the presence of the macrocycle is indeed what leads to the initial inhibition of the Pt^{II}-salphen.

We were interested in further investigating the cellular localisation of **46**^{photo} and how it might change upon irradiation. To this end, additional live cell co-localisation experiments were carried out with **46**^{photo} in which LysoTracker Green and DAPI were added; this confirmed that there was no nuclear staining and that the compound did not localise in the lysosomes. Although no further co-staining experiments were carried out, one potential site may be the mitochondria as quaternary ammonium groups have been used as mitochondria-targeting moieties. We then attempted to carry out some live cell imaging with U2OS cells which had been incubated with **46**^{photo} and irradiated. However, significant levels of cell death were seen. This meant that without considerable optimisation of conditions it would be difficult to ascribe a change in localisation specifically to Pt^{II}-salphen release as many types of compounds localise in the nucleus of dead cells. We therefore moved onto fixed cell imaging.

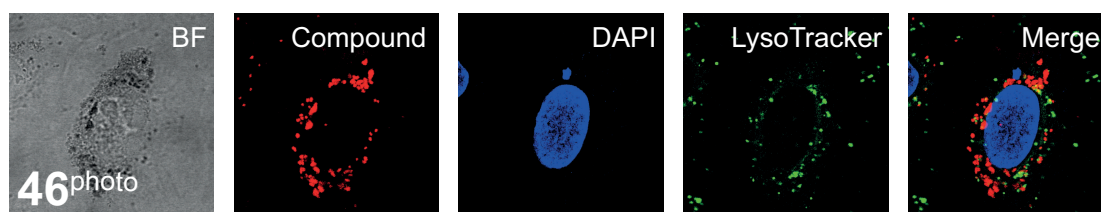


Figure 4.18 Live cell fluorescence imaging experiments with **46**^{photo}, including co-staining with DAPI and LysoTracker Green. Pt^{II}-salphen $\lambda_{\text{ex}} = 458 \text{ nm}$, $\lambda_{\text{em}} = 525\text{--}700 \text{ nm}$, DAPI $\lambda_{\text{ex}} = 760 \text{ nm}$ (2 photon), $\lambda_{\text{em}} = 400\text{--}450 \text{ nm}$. LysoTracker Green $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 570\text{--}700 \text{ nm}$.

For fixed cell experiments U2OS cells were seeded and grown for 24 hours, followed by addition of 2 μM of **46**^{photo} or **46**^{ctrl} and incubation for a further 24 hours. The cells were then washed and fixed with PFA, before either being left in the dark or irradiated for 20 minutes. Finally, DAPI was added as a co-stain and the cells were imaged. As can be seen from Figure 4.19, under fixed cell conditions **46**^{photo} displayed a similar pattern of localisation seen in live cells, with punctuate non-nuclear localisation. However, after ir-

radiation the localisation pattern was completely transformed, showing primarily nuclear localisation and good overlap with DAPI caused by an increased affinity to DNA. These experiments correspond well with the DNA binding experiments discussed in Section 4.3. Importantly, no corresponding change in nuclear localisation was seen for the analogous experiment conducted with **46**^{ctrl}.

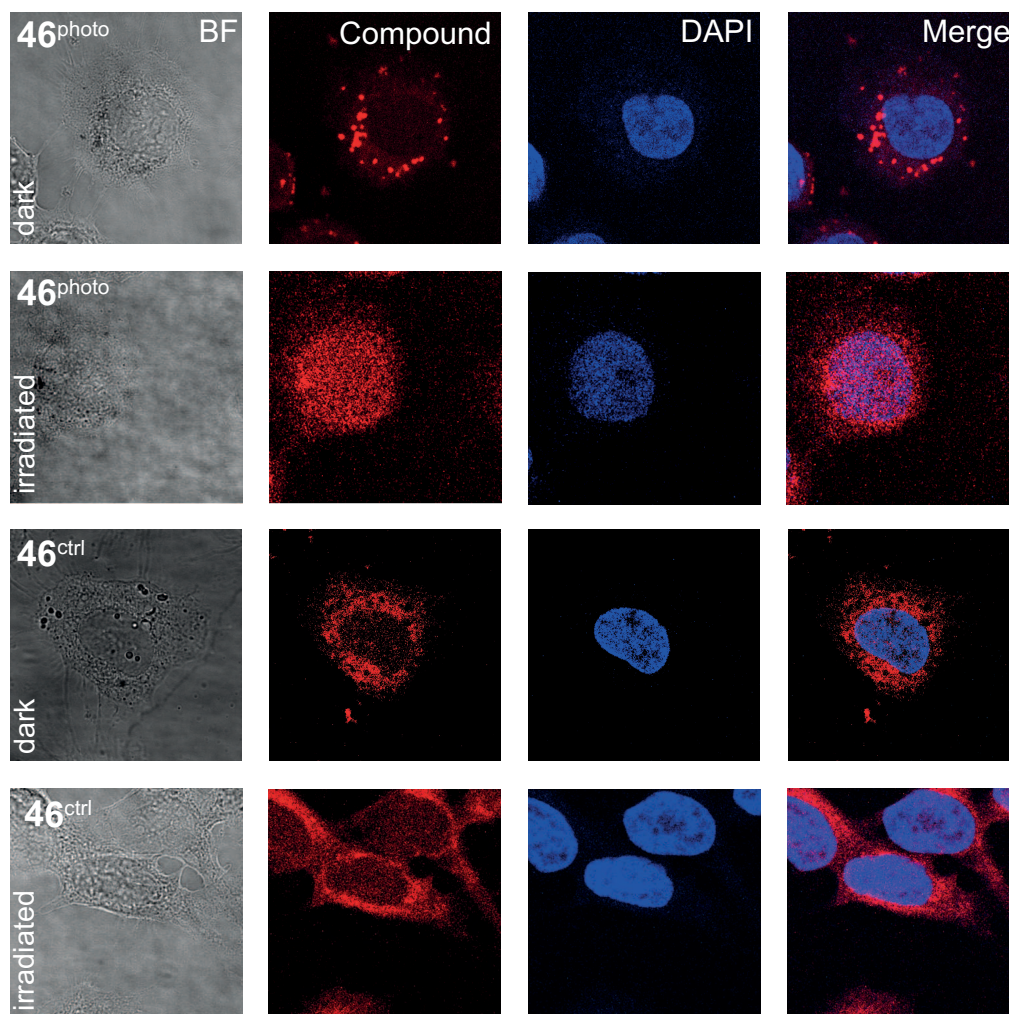


Figure 4.19 Fixed cell imaging experiments with rotaxanes **46**^{photo} and **46**^{ctrl} (2 μ M for 24 hours) in cells which were either left in the dark or irradiated for 20 minutes at 365 nm. DAPI was added for nuclear staining. Pt^{II}-salphen λ_{ex} = 458 nm, λ_{em} = 525-700 nm, DAPI λ_{ex} = 760 nm (2 photon), λ_{em} = 400-450 nm. All images are to the same scale.

4.6 Conclusions and future work

In conclusion, we have synthesised a new family of interlocked molecules containing a shielded DNA-targeting Pt^{II} -salphen. The presence of the macrocycle effectively blocks any interactions between the Pt^{II} -salphen and DNA as evidenced by fluorescence titrations and CD melting experiments. However, by including a two-stage cleavable stopper in the rotaxane, we are able to trigger the release of the Pt^{II} -salphen and restore its ability to stabilise G4 DNA. Both autonomous activation via esterase and photo-activation via irradiation have been tested.

Furthermore, the rotaxanes were able to transport the non-cell permeable Pt^{II} -salphen into cancer cells in which they remained non-cytotoxic until triggered release. In the case of **46**^{enz}, this appeared to occur on its own, offering a potential mechanism for controlled autonomous gradual dosing of a drug. For **46**^{photo}, the interlocked cage was stable for up to 24 hours (the longest time period tested) but could be triggered to release the Pt^{II} -salphen in a highly controlled manner. The release of the Pt^{II} -salphen caused significant cell death presumed to be due to interactions with DNA in the nucleus. This was further corroborated by fixed cell imaging experiments which showed changes in localisation of the compound upon irradiation and release. This approach, in which rotaxanes can be used to control the bioactivity of a small molecule, paves the way for a new generation of smart drugs with controlled targeting and activation by external stimuli.

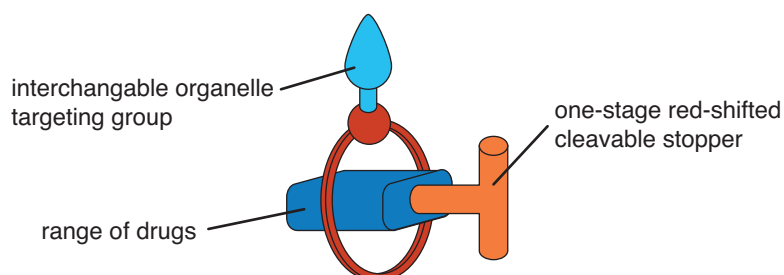


Figure 4.20 A potential second-generation interlocked drug carrier.

Indeed, we intend to continue this work and extend its applications. Firstly, it will be important to demonstrate that it can be widely applied to a range of drugs with varying targets. Alongside this, the biological activity and effect of the molecular cages themselves will

need to be investigated in much more detail. We also wish to investigate whether the cellular localisation can be controlled on a sub-cellular level via the addition of organelle targeting groups, specifically, whether this can be included in the modular design approach. Finally, we wish to address some limitations of the current work; namely that UV irradiation can damage cells significantly. We will therefore aim to switch to a more red-shifted activation wavelength, of which a number of potential fluorophores have already been reported. A schematic of a potential second-generation probe is shown in Figure 4.20.

Chapter 5

A redox caged Pt^{II} -salphen

5.1 Background

An exciting feature of some metal-based drugs and optical probes is that the redox state of the metal centre can be varied. The resulting change in the d electron count of the metal centre can be accompanied by shifts in reactivity, geometry or the emissive properties of the complex.^[231,232] In a biological context, redox-active metal complexes can be used to influence the redox potential in cells directly or interact with biomolecules which are involved in cellular redox pathways.

Redox homeostasis plays a hugely important role in the regulation of cell survival.^[233–236] Under normal physiologic conditions, redox potential is controlled through the generation and elimination of reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, cells can enter oxidative stress when redox homeostasis becomes unbalanced, which has been linked to cancer, neurodegenerative diseases and ageing. For example, cancer cells often have increased levels of ROS generation, associated with increased expression of certain oncogenes including Ras and Myc. This higher state of oxida-

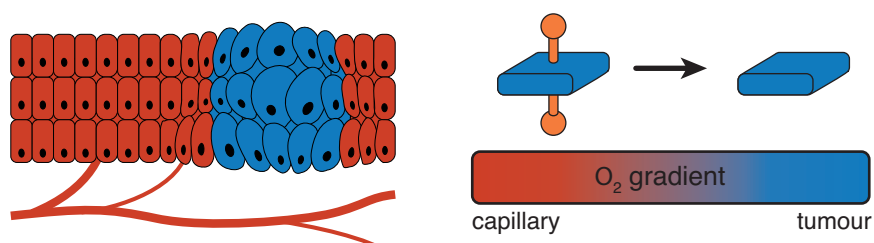


Figure 5.1 Cancer tumours exist in a hypoxic state with higher levels of oxidative stress. Hypoxia-activated prodrugs utilise the associated reducing environment to specifically become activated in the tumour microenvironment.

tive stress is thought to activate cell survival pathways, promote growth, and help avoid cell senescence. Another consequence of the aberrant growth of cancerous cells is that the rate of proliferation outpaces the ability of the body to supply oxygen to the tumour microenvironment. This leads to a low oxygen environment known as hypoxia, placing cells under a high energy demand with limited glucose supply. This can lead to oxidative stress through reduced oxygen radicals decomposition.^[237,238] The hypoxic and oxidative stress found in tumours has been linked to increased levels of bioreductants such as glutathione as well as the availability of oxygen-inhibited enzymatic reduction pathways.^[239] Hypoxia-activated prodrugs aim to utilise these mechanisms for activation solely in the tumour microenvironment.^[240]

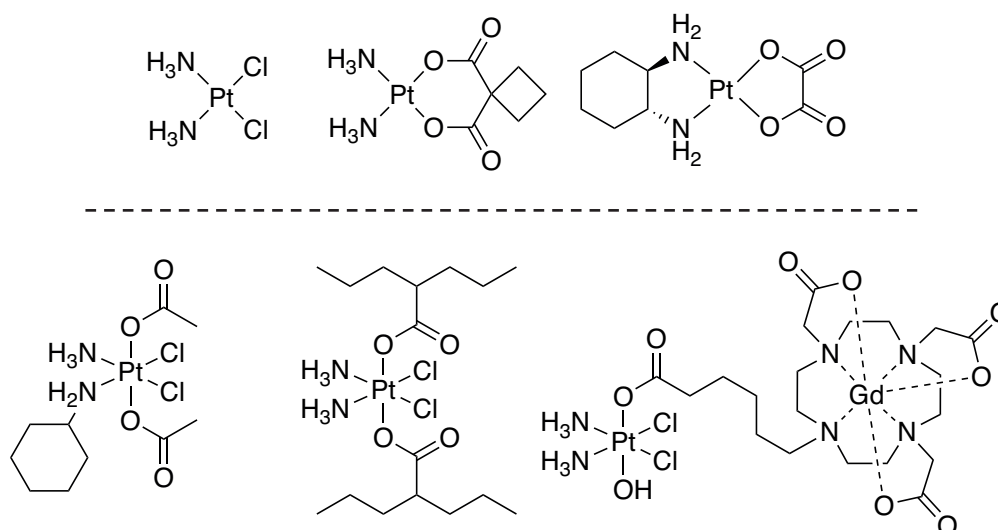


Figure 5.2 Pt^{II} metallodrugs cisplatin, carboplatin and oxaliplatin and some examples of Pt^{IV} prodrugs with different axial ligands for dual dosing or imaging capabilities.

Some of the most well-studied hypoxia-activated prodrugs are Pt^{IV} complexes primarily based around the Pt^{II} complex cisplatin and its analogues.^[241] Since the discovery of cisplatin in the 1960's, cisplatin, carboplatin and oxaliplatin (Figure 5.2) have accounted for a large proportion of chemotherapy treatments in which they are thought to act through DNA damage *via* ligand substitution and DNA linking.^[242] Pt^{IV} analogues of these complexes fall into the category of prodrugs as they are much more inert and resistant to ligand substitution than their Pt^{II} counterparts. The chemistry and available functionality of

Pt^{IV} prodrugs is rich and through judicious choice of the two axial ligands lipophilicity, redox potential, fluorescence or target specificity can be modified. For example, satraplatin is a Pt^{IV} prodrug with a number of potential advantages over cisplatin including oral administration and a milder toxicity profile currently in clinical trials.^[243] Alternatively, a dual action approach can be pursued. Combining cisplatin with compounds known to increase chromatin openness (such as the histone deacetylase (HDAC) inhibitor valproate) can lead to up to a 100-fold increase in potency. Upon activation, the active form of both drugs are released.^[244] Alternatively, imaging agents such as gadolinium can be added in the axial position, leading to a complex with both therapeutic and diagnostic capabilities.^[245] The axial ligands can also be used to attach Pt^{IV} prodrugs to the surface of nanoparticles.^[246]

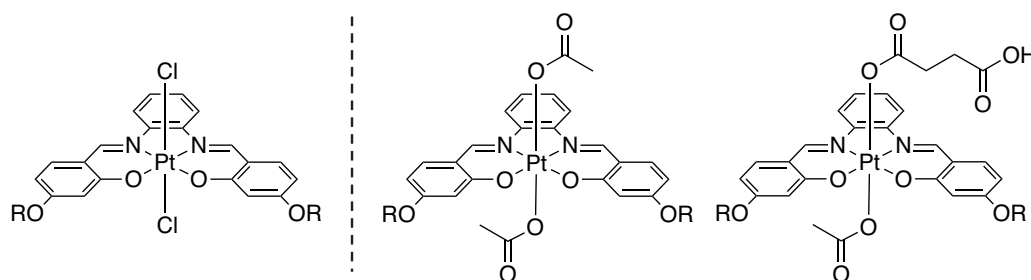


Figure 5.3 The first generation Pt^{IV} -salphen with chlorido ligands (left) and our new targets with carboxylato ligands (right).

Our group has been interested in the application of hypoxia activated Pt^{IV} prodrugs to metal complexes which target G4 DNA. In particular, we have looked at applying this approach to Pt^{II} -salphens. As discussed in the previous Chapters, having the aromatic surface of the metal salphen unhindered is essential for effective stabilisation of G4 DNA. The square planar Pt^{II} metal ion lies in the centre of the surrounding Schiff base. Consequently if this is oxidised to a Pt^{IV} centre and the geometry is switched to octahedral the axial ligands should provide a very effective block providing that they have sufficient bulk (as mentioned previously, some metal salphen complexes with axial ligands such as ammonia can stabilise G4 DNA through interaction with the central ion channel, meaning that the aromatic core can still $\pi - \pi$ stack with the outer tetrads). In 2017 a first-generation Pt^{IV} -salphen was synthesised with chlorido axial ligands.^[172] Whilst this compound showed significant promise in early tests, it was not suitable for cellular studies as it was very quickly

reduced when incubated in cell media. We therefore aimed to synthesise a set of second generation of Pt^{IV} compounds with improved stability, focussing on the use of carboxylato ligands.

This Chapter is split into the following parts: (1) synthesis and characterisation of a new series of Pt^{IV} -salphens; (2) interaction of the Pt^{IV} -salphens with DNA; (3) investigation into activation and suitability of these complexes for use in cells.

5.2 Synthesis

5.2.1 Compound design

The most common form of Pt^{IV} prodrug synthesis is via a two-electron oxidation of the Pt^{II} compound.^[247] Some common oxidising agents are shown in Figure 5.4. Depending on the conditions and solvent used, various axial ligands can be added. For example, using dichloriodobenzene and N-chlorosuccinimide, either one or two chlorido axial ligands can be added. Similarly, hydrogen peroxide can be used to add hydroxido axial ligands or a combination of hydroxido and carboxylato ligands. Another common oxidising agent is diacetoxyiodobenzene, which has been utilised to add two carboxylato ligands. Furthermore, once formed, some Pt^{IV} compounds are suitable for post-synthetic modification; for example Pt^{IV} compounds with hydroxyido ligands can be combined with succinic anhydride in order to obtain a reactive handle to which many further modifications can be made.

In addition to providing potential for further modification, the identity of the axial ligands plays a crucial role in the biological activity of the compounds. This is primarily due to the reduction potential of the Pt^{IV} complex being highly dependent on which axial ligand is present as well as the overall effect on lipophilicity. Generally, Pt^{IV} compounds with chlorido axial ligands are the most easily reduced, with cisplatin analogues having a reduction potential of around -250 mV followed by carboxylato at around -600–700 mV and hydroxido at -900 mV (all v.s. Ag/AgCl). Whilst it may seem like there should be a correlation between the biological effectiveness of Pt^{IV} prodrugs and the reduction potential, such a link has been difficult to find.^[248,249] Although no direct link to reduction potential has

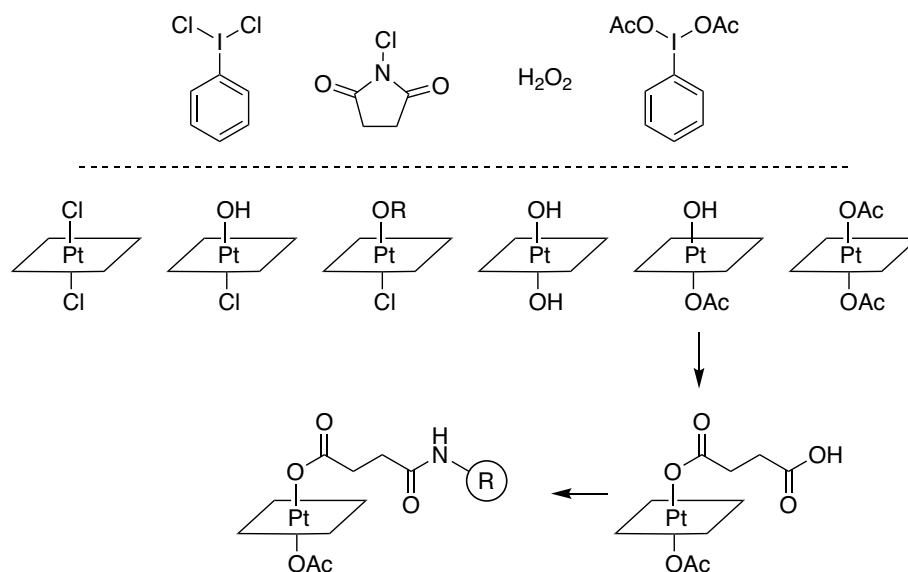


Figure 5.4 Oxidising agents used to prepare Pt^{IV} prodrugs with a range of axial ligands and their subsequent modification.

been found, the purpose of the axial ligands is to allow for accumulation of the compounds in the tumour microenvironment and to undergo reduction to release their cytotoxic cargo; therefore they must be stable enough to reach the tumours but labile enough for some level of reduction to occur.

Initial work by our group looked at a number of different oxidising agents to see whether they could be suitable for Pt^{IV} -salphen synthesis. As has been shown previously, the use of aromatic multi-dentate ligands changes the HOMO and LUMO energies as well as their distribution which can change their reactivity. Ravera and coworkers demonstrated that in Pt^{II} -terpy complexes the LUMO is delocalised over the terpy ligand, with very little density at Pt^{II} centre, making its reaction with hydrogen peroxide less favoured.^[250] In addition, it has been reported that under some conditions many side products can be formed. The best results obtained by our group was using the hypervalent iodine complex dichlorido iodobenzene, and whilst a pure compound was obtained, reproducibility proved to be a major issue. The use of hydrogen peroxide and N-chlorosuccinimide was also attempted but was ultimately unsuccessful.

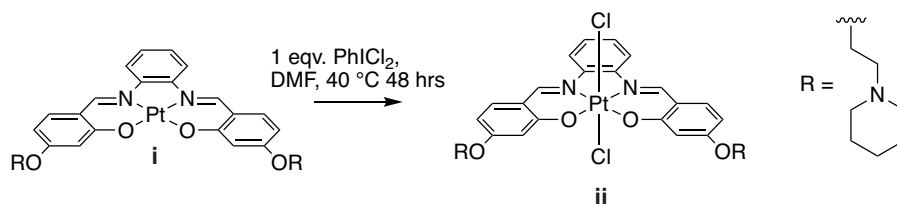


Figure 5.5 The first generation Pt^{IV} -salphen with chloride ligands synthesised with a hypervalent iodine compound

Initial *in vitro* results were promising; the Pt^{IV} compound **ii** had a reduction potential of -216 mV and could be activated via incubation with both ascorbic acid and glutathione. Additionally, CD melting experiments showed that the Pt^{IV} -salphen did not stabilise either c-Myc or duplex DNA. However, unfortunately, when the compounds were incubated with BSA or cell culture media containing as low as 0.1% FBS very rapid reduction was observed, with a half life of 5 minutes. This made the compounds unsuitable for further studies.

We therefore aimed to develop a new synthetic route to Pt^{IV} -salphen complexes. We chose to focus on the use of hypervalent iodine oxidising agents as they had shown promise in the initial work. Diacetoxyiodobenzene (DIB) is hypervalent iodine compound which has been used to synthesise Pt^{IV} compounds with two carboxylato ligands, which have significantly higher reduction potentials than their chlorido counterparts. Additionally, whilst the initial tests had been carried out with the Pt^{II} -salphen with piperidine substituents (compound **i**, renumbered **49**), we had synthesised a range of compounds with trimethylammonium functionalised side-chains (re-numbered in this Chapter as compound **48** for clarity), of which the isolation of the hexafluorophosphate salt gave us access to a slightly wider range of solvents compared with the neutral piperidine analogue (compound **i**, renumbered **49**).

In particular, a literature search had showed that oxidising reactions carried out with diacetoxyiodobenzene had often used DCM or acetonitrile as the solvent. Whilst **48** was not soluble in DCM, it was soluble in acetonitrile. Additionally, as acetonitrile- d_3 is readily available, we would be able to track the reaction *in situ*. First, ^1H NMR spectra of the Pt^{II} -salphen starting material **48**, DIB and iodobenzene (the byproduct of the oxidation) were recorded for reference and to track whether the oxidising agent was being consumed

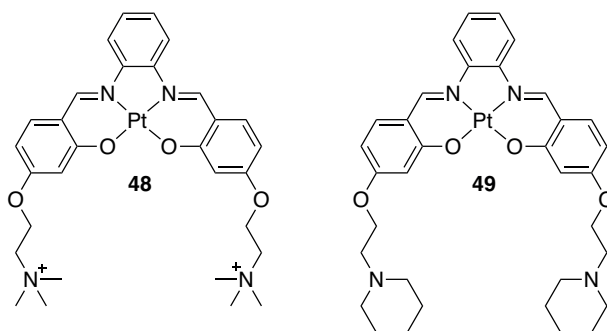


Figure 5.6 Pt^{II} -salphen compounds investigated in this Chapter.

during the reaction. The ^1H NMR spectra of Pt^{II} -salphen **48** displays six easily identifiable proton environments, whilst the DIB and iodobenzene display the 2/1/2 pattern of doublets and triplets characteristic of singly substituted benzene derivatives.

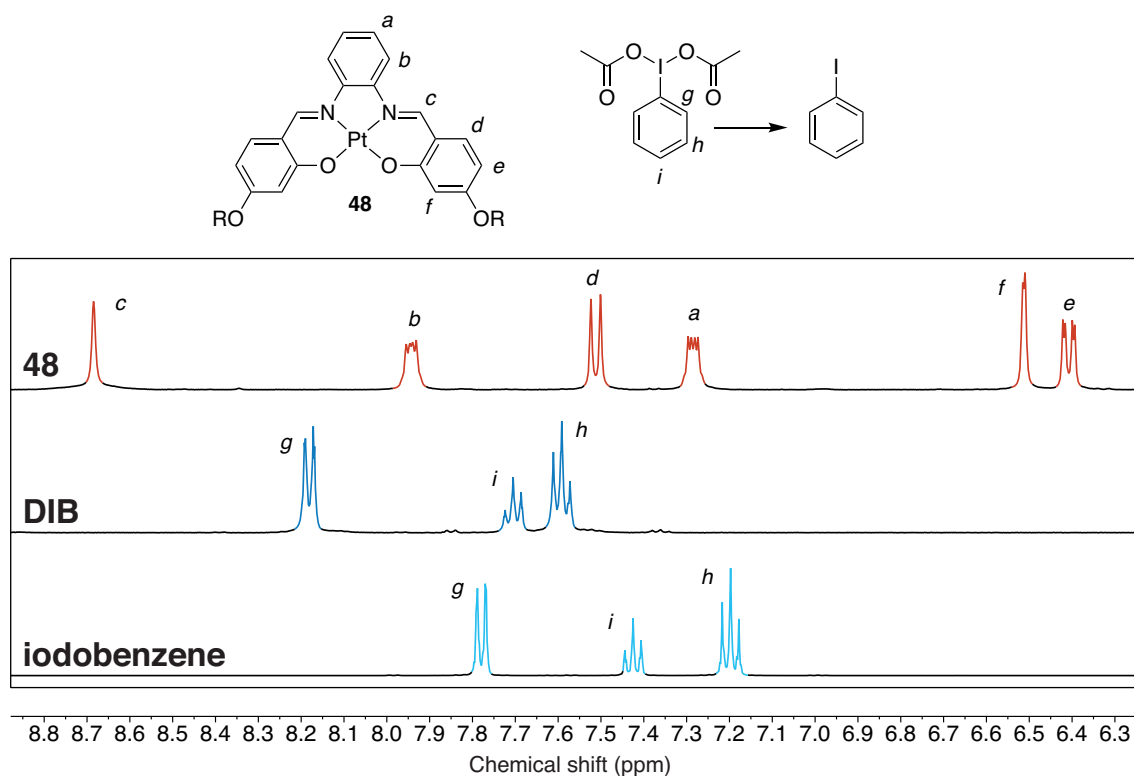


Figure 5.7 ^1H NMR spectra of **48**, DIB, and iodobenzene.

With the reference spectra in hand, 10 mg of **48** was dissolved in acetonitrile- d_3 and an excess (4 equivalents) of oxidising agent was added. ^1H NMR spectra were recorded at 1, 4, 16 and 24 hours (shown in Figure 5.8). The imine peak has been highlighted as the chemical shift of this proton is a sufficient distance from the rest in order for it to be easily tracked. Additionally, it is the proton closest to the platinum centre and undergoes the

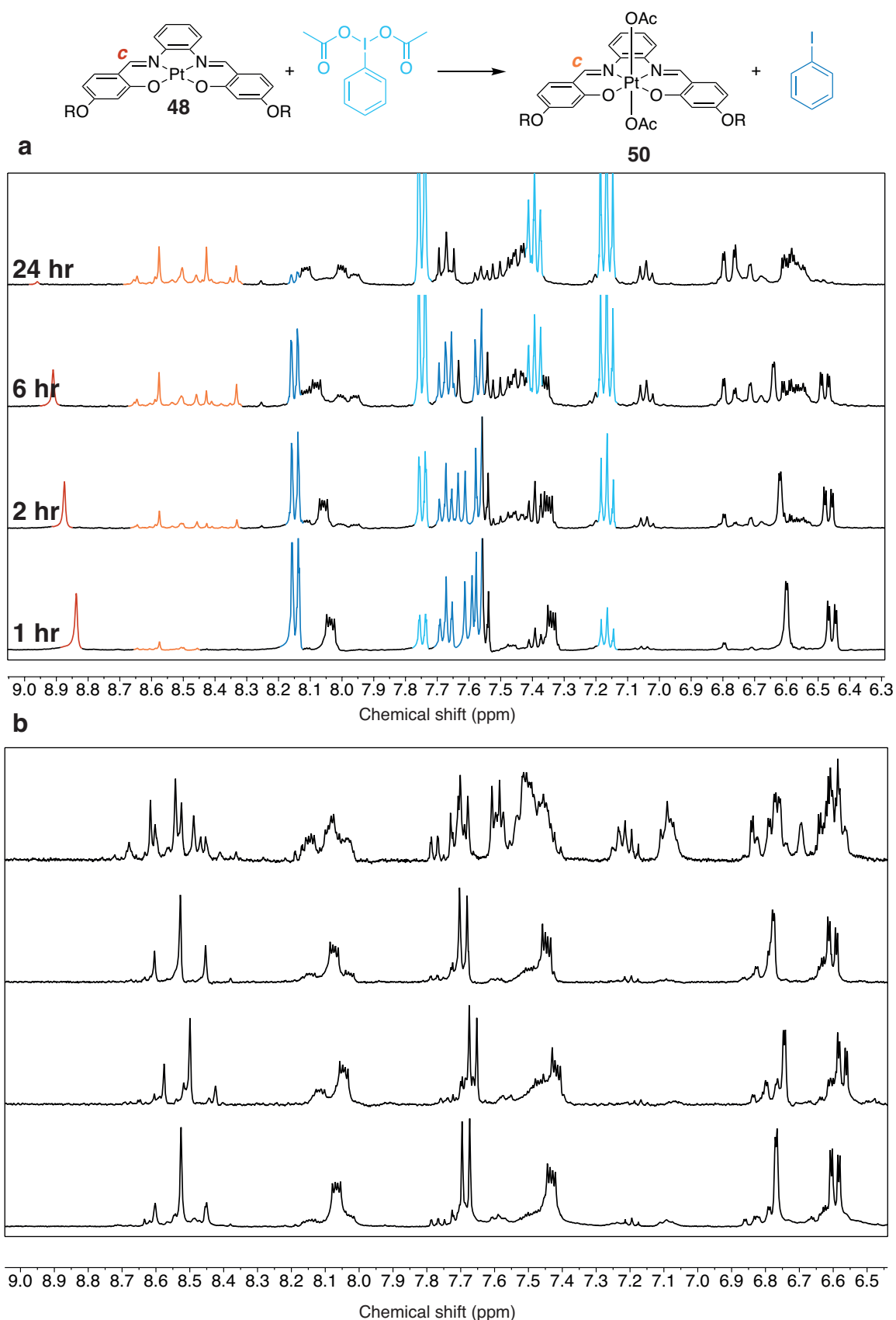


Figure 5.8 ^1H NMR experiments: (a) tracking a single oxidation reaction via ^1H NMR and (b) multiple oxidation reaction attempts using identical conditions but showing significantly different levels of purity.

largest shift upon Pt^{IV} formation. This peak should also display satellite peaks from coupling to the ¹⁹⁵Pt (*I* = 1/2, 33% abundance). As can be seen, the imine proton corresponding to the starting material shifted over 24 hours, with a number of new peaks appearing in the range of 8.4-8.7 ppm. Concurrently, the peaks corresponding to DIB disappeared and peaks corresponding to iodobenzene appeared. Finally, a colour change observable to the naked eye from orange to yellow was seen and analysis using a long wave UV lamp (365 nm) showed that the sample was no longer emissive, in line with previous reports showing that while the Pt^{II} complex is emissive, the Pt^{IV} derivate is not.^[172] However, whilst no starting material remained, it was also evident that the reaction had not proceeded cleanly and many products had formed.

This process was subsequently repeated multiple times. In order to better analyse the NMR, after the reaction mixture was no longer changing the reaction was worked up, following a standard procedure in which the iodobenzene and any excess oxidising agent was removed via precipitation of the product with diethyl ether. Unfortunately, as can be seen from Figure 5.9b, reactions carried out under supposedly identical conditions gave quite different product distributions and levels of purity. Further purification of the cleanest crude reaction mixture was attempted via recrystallisation with acetonitrile/diethyl ether; and is shown in Figure 5.9.

As can be seen a single product was isolated, showing clear ¹⁹⁵Pt-¹H coupling between the metal centre and the imine proton. The ¹⁹⁵Pt NMR spectrum shows a single peak at 1000 ppm, representing a significant shift from the starting material (-1200 ppm) and in the range of other Pt^{IV} compounds with dicarboxylate ligands. Similar to the dichlorido complex, the ¹⁹⁵Pt chemical shift is slightly more upfield than the corresponding cisplatin Pt^{IV}-carboxylate prodrug complex.^[251,252] Finally, a 2D HMQC NMR experiments confirmed cross-peaks between the imine satellites and the Pt peak. Mass spectrometry was used to confirm the formation of the Pt-salphen with two coordinated acetate groups.

Whilst this data was promising, two major problems remained. Firstly, the recrystallisation process was difficult to reproduce and coupled with the unreliability of the oxidation

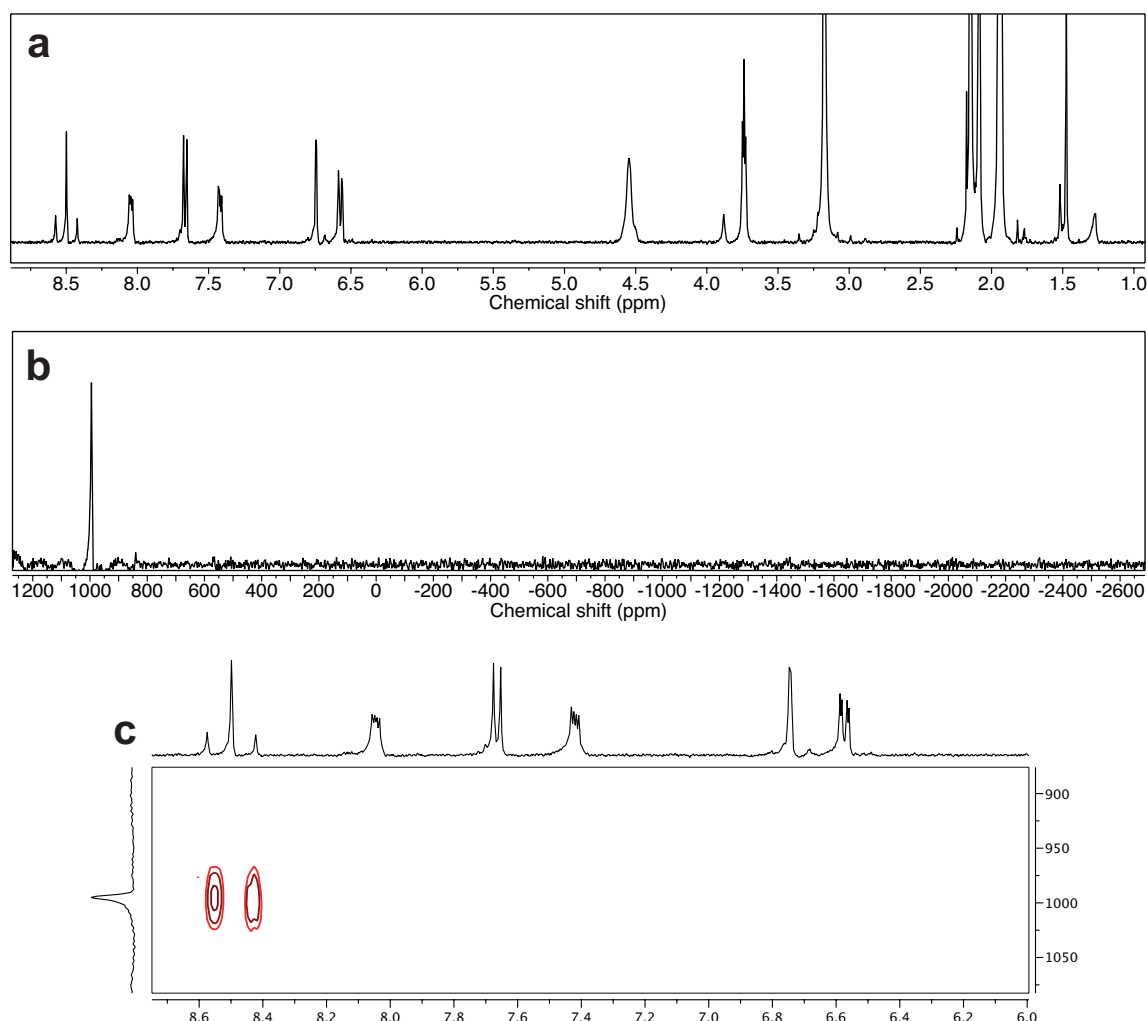


Figure 5.9 Pt^{IV} -salphen characterisation: (a) ^1H spectrum of **50**, (b) ^{195}Pt NMR spectrum and (c) 2D NMR showing coupling between imine satellites.

step made repeating/scaling up this reaction very difficult. Secondly, data had emerged from other experiments which showed that a number of metal salphen complexes (both square planar and octahedral) with trimethylammonium side-chains displayed very poor cellular uptake. Therefore, we decided to attempt to optimise the reaction conditions, but with the piperidine functionalised complex.

5.2.2 Towards a multi-functional dicarboxylate Pt^{IV} -salphen

When carrying out the oxidation experiments, LCMS was used to analyse the purity of the reaction mixtures. Often, we observed a mass corresponding to the mono-carboxylato/mono-hydroxido product alongside the dicarboxylato product. We therefore

had the idea to see if we could change the reaction conditions to favour the mono-carboxylato/mono-hydroxido product rather than trying to form the dicarboxylato compound as the major product (Figure 5.10). Firstly, the piperidine functionalised Pt^{II}-salphen was isolated as the positively charged hexafluorophosphate salt via dissolving the compound in water (in which it was partially soluble), followed by the addition of 2M HCl until the compound was fully dissolved. Next, a saturated solution of NaPF₆ was added, causing the compound to precipitate out of solution, at which point it was filtered and washed with a further quantity of water. Next, a modified oxidation reaction was carried out, in which the diacetoxyiodobenzene was first dissolved in acetonitrile, before an equal quantity of water was added. A solution of the Pt^{II}-salphen in acetonitrile was then added drop-wise. Again, a clear colour change from orange to yellow was observed, and after 2-3 hours the reaction was stopped, the acetonitrile was removed via rotary evaporation and the compound precipitated with sodium hexafluorophosphate.

Pleasingly, ¹H NMR analysis revealed only one peak corresponding to the imine protons, and again Pt satellites were clearly observable. However, there were a number of additional peaks. Due to the overlapping chemical shifts it was difficult to fully confirm integrations and separate environments. However, it appeared that there were two sets of a doublet/triplet/triplet pattern. Comparison with the ¹HNMR chemical shifts of other potential reactants and products (iodobenzene and diacetoxyiodobenzene) revealed that one of these sets of doublet/triplet/triplets was from iodobenzene (shown in light blue) and confirmed that the other set was not due to unreacted diacetoxyiodobenzene. Additionally, this experiment was conducted multiple times and the ratio of the peak integrations was roughly consistent with a 1:1 ratio between the Pt^{IV}-salphen formed and one molecule of a singly substituted benzene.

We therefore considered what other products could be forming. A number of papers have investigated the mechanism by which hypervalent iodine compounds take part in oxidation reactions; generally it is assumed that the HOMO on the reactant can attack the hypervalent iodine, in this case forming a 5-coordinate Pt^{IV} compound.^[253,254] At this point, a

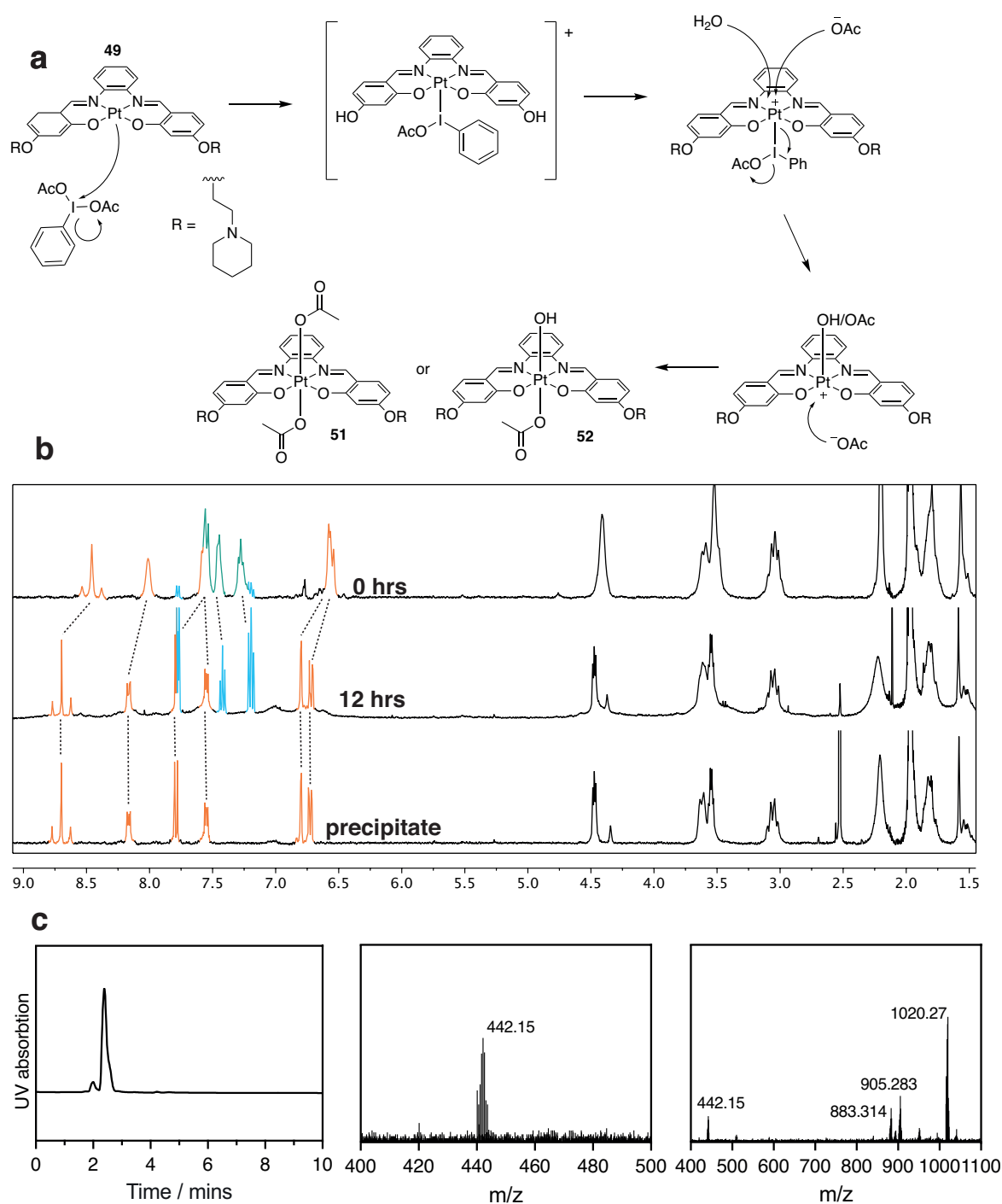


Figure 5.10 Pt^{IV} -salphen synthesis: (a) Potential mechanism of the formation of either carboxylato or hydroxido Pt^{IV} -salphens, (b) ^1H NMR data of the Pt^{IV} -salphen intermediate left in acetonitrile and then precipitated out to give the final product Pt^{IV} -salphen **51** (top to bottom) and (c) LCMS data of **51**.

solvent molecule could coordinate to the empty coordination site, causing ligand exchange and eventual loss of iodobenzene with exchange to the carboxylate product. However, it could be that this intermediate complex in which the iodobenzene is still coordinated to the metal is formed, which would explain the extra peaks found in the NMR. When this product was left in acetonitrile- d_3 , a further reaction took place. A shift in each proton corresponding to the Pt^{IV} -salphen was seen, accompanied by transformation of the proposed bound iodobenzene to shifts corresponding to free iodobenzene, providing further evidence that it might be the bound intermediate that we had previously isolated. We decided to leave the reaction overnight until full product transformation had occurred, before precipitating and washing with diethyl ether to remove the excess remaining iodobenzene.

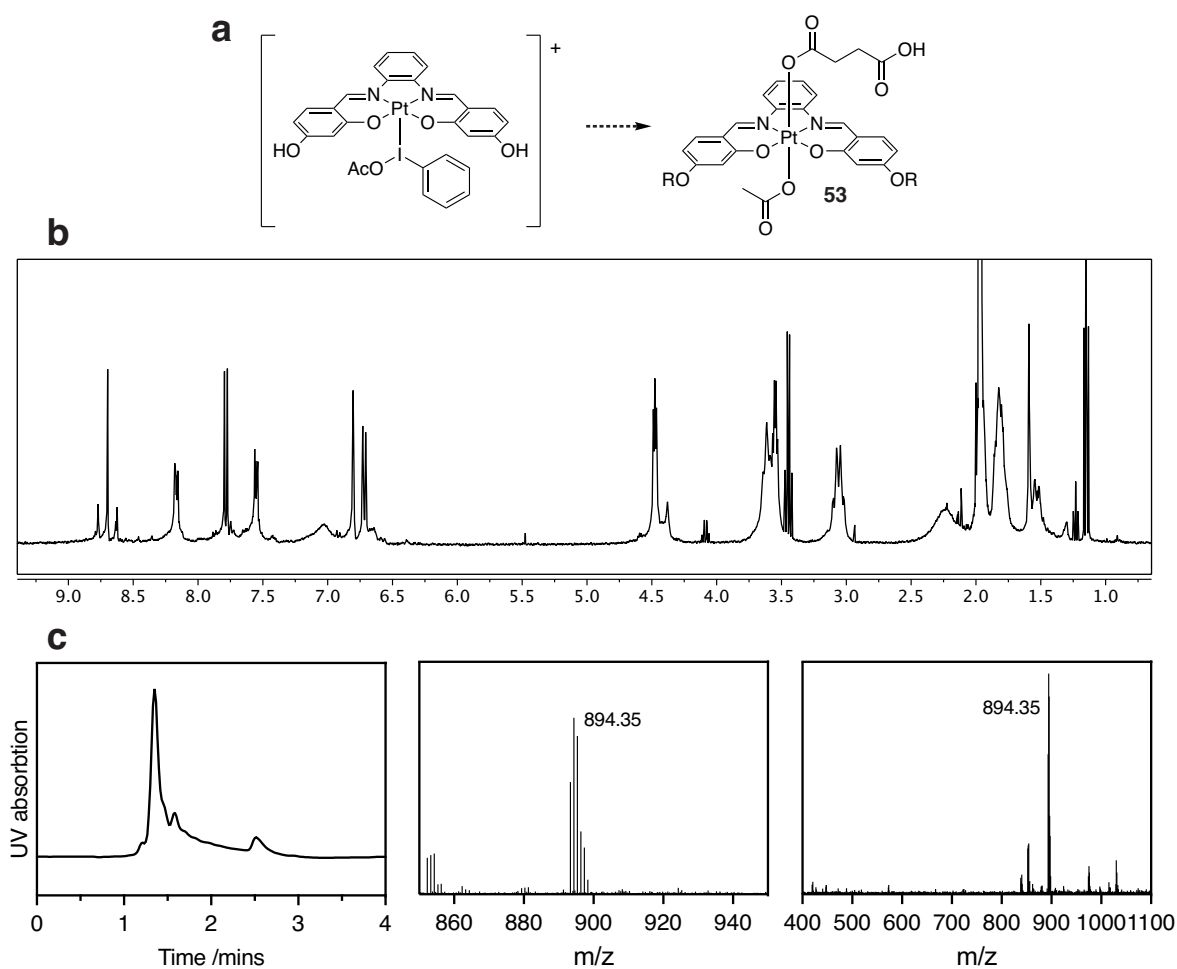


Figure 5.11 Attempted formation of Pt^{IV} -salphen **53**: (a) reaction mechanism, (b) ^1H NMR spectrum and (c) LCMS data.

¹H NMR analysis alongside LCMS showed that the compound was 95%+ pure. High resolution mass spectrometry indicated that the dicarboxylato product (compound **51**) had formed; however, without further evidence we are unsure as to what exact mechanism could lead to the formation of this product.

We also carried out a further reaction with the compound which appeared to be the intermediate Pt^{IV}-salphen, with the aim of forming a carboxylic-functionalised handle (Figure 5.11). In this case, the compound was dissolved in DMF and heated at 80 °C overnight with succinic anhydride. The ¹H NMR showed the formation of a major product. However the correct mass could not be identified via HRMS. Additionally, there was a reasonable amount of impurity present so we decided to move forward to DNA binding studies with the diacetoxido product **51**.

5.3 DNA Binding

With evidence in hand of the formation of a dicarboxylate Pt^{IV}-salphen (**51**), we moved on to compare its DNA binding properties with its Pt^{II} analogue (compound **49**). Fluorescence titrations could not be carried out in this case due to the Pt^{IV}-salphen being non-emissive. Therefore a fluorescence indicator displacement assay was used. This assay works by pre-incubating a known DNA binder (in this case thiazole orange, or TO) with the DNA structure of interest. Then, increasing quantities of the ligand under study are titrated against the TO/DNA. If the ligand binds effectively to DNA, the TO is displaced and its emission switched off, whereas if it is a poor DNA binder the TO will stay bound and little change will be seen. For this experiment, 1 μM c-Myc G4 DNA was incubated with 2 μM TO, followed by the addition of 0–10 μM (or 0–10 equivalents relative to DNA) of either Pt^{II}- or Pt^{IV}-salphen. Percentage displacement curves were calculated from the measured fluorescence intensity (F), using: displacement = 100 - [(F/F₀) × 100], where F₀ is TO fluorescence from the probe bound to c-Myc without the added compound. Displacements were fitted to a Hill function which was used to calculate the DC50. The results are shown in Figure 5.12a; Pt^{II}-salphen **49** displaced TO rapidly, with a DC50 value of 1.3 μM. In comparison,

Pt^{IV} -salphen **51** never reached 50% displacement, even at 10 equivalents of ligand, demonstrating that the axial ligands effectively block any G4 binding.

Next, a series of melting experiments were conducted. Firstly, CD melting was used to compare how well the Pt^{II} - and Pt^{IV} -salphen compounds could stabilise c-Myc G4 DNA in order to corroborate the FID assay. These experiments were carried out in an analogous manner to before in which 5 μM DNA was incubated with 10 μM compound and the temperature varied between 25–95 $^{\circ}\text{C}$. The results are shown in Figure 5.12b; addition of Pt^{IV} -

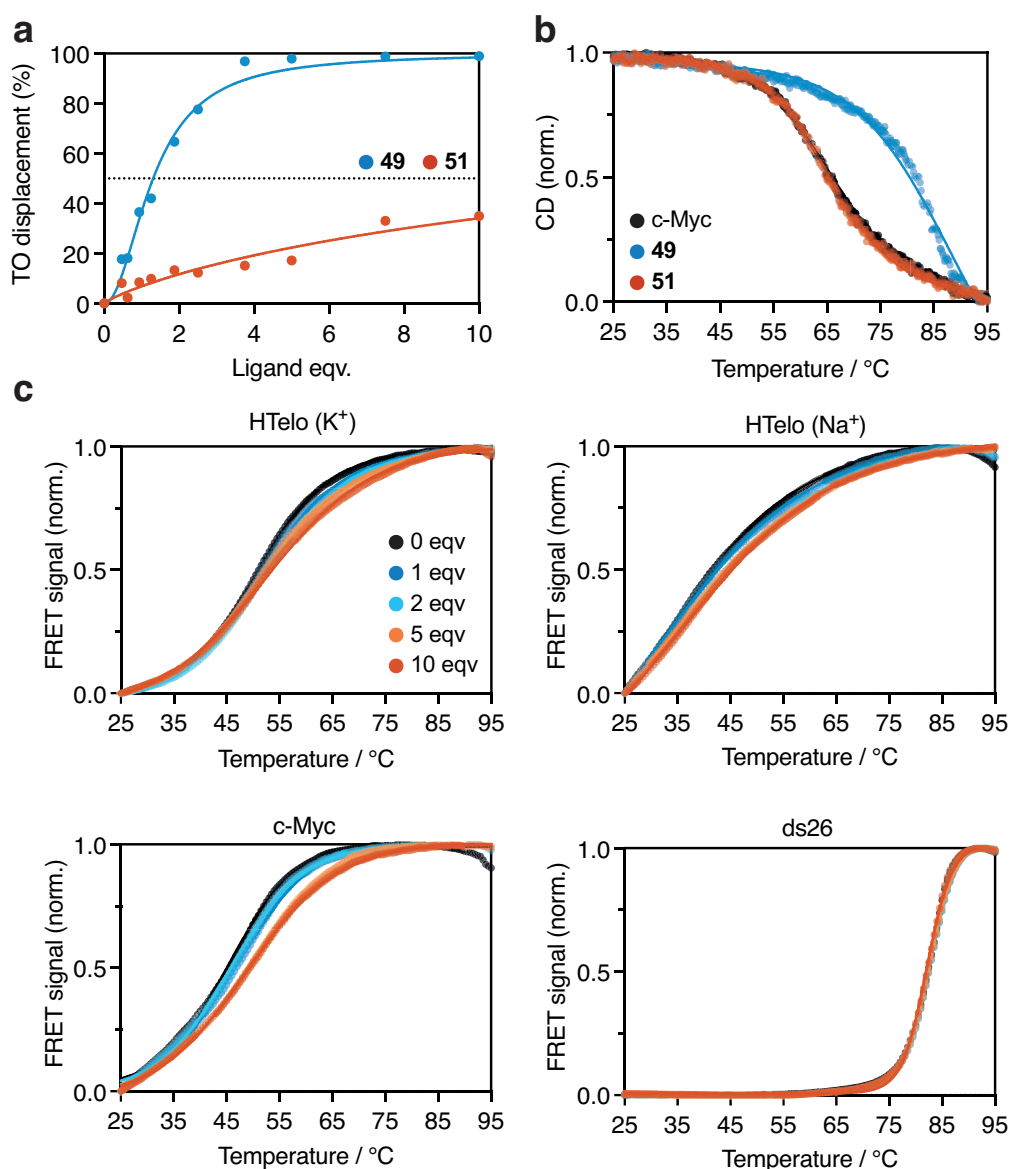


Figure 5.12 DNA binding experiments comparing Pt^{II} -salphen **49** with Pt^{IV} -salphen **51**: (a) FID assay with thiazole orange and c-Myc G4 DNA, (b) CD melting with c-Myc G4 DNA and (c) FRET melting experiments with various DNA sequences.

salphen **51** led to no discernible change in DNA melting temperature whereas the addition of **49** led to an 18 °C increase.

Finally, FRET melting was used to test the stabilising effects of Pt^{IV}-salphen **51** against a wider range of DNA topologies. FRET melting is a technique which utilises a pair of fluorophores (A and B) in which the emission of A overlaps with the absorption of B. This allows for an energy transfer process (non-radiative dipole-dipole coupling) to take place in which emission is only observed from fluorophore B. The efficiency of this process is proportional to the inverse of the sixth power of the distance between the two fluorophores, making it a very sensitive technique. In this case, the conformation of the folded DNA leads to the two fluorophores being near each other and the FRET process occurring. During a melting experiment the temperature is increased, leading to the DNA strand unfolding and the distance between the fluorophores increasing. This can therefore be followed by track the emission of either fluorophore; the emission of fluorophore A will increase whilst the emission of B will decrease.

FRET melting had not previously been used to investigate Pt^{II}-salphen–DNA interactions due to the absorption and emission of the Pt^{II}-salphen overlapping with the wavelengths needed to monitor the fluorophores of the labelled DNA under study. However, as Pt^{IV}-salphen **51** was non-emissive, it could be used. Four DNA topologies were tested: c-Myc, HTelo (Na⁺), HTelo (K⁺) and a duplex sequence ds26. In this experiment, 0.2 μM of DNA was combined with 0, 1, 2, 5 and 10 equivalents of compound and the melting temperature was measured. The two fluorophores used were 6-FAM (fluorophore A) and TAMRA (fluorophore B) and the emission of 6-FAM was tracked. Conventionally the degree of thermal stabilisation is quoted as the value obtained from the addition of 1 μM (5 equivalents) of compound. Across all four DNA structures only minimal stabilisation of 1-4 °C was observed. In comparison, the Ni^{II} analogue of compound **49** has been shown to stabilise G4 DNA by up to 30 °C.

Taken in concert, the FID and melting assays clearly show that the Pt^{IV}-salphen has very poor affinity for G4 and duplex DNA. We therefore moved on to investigate whether they

offered improved stability and therefore potential to be used in cellular assays.

5.4 Stability and activation

Firstly, the ability of a range of bio-reductants to reduce the Pt^{IV}-salphen were tested. Previously, it had been shown that both sodium ascorbate and L-glutathione were able to reduce the dichlorido Pt^{IV}-salphen complex. In a mixture of DMSO-D₂O, using a 1:2 molar ratio (Pt^{IV}/GSH), full reduction was observed in under 10 minutes. With sodium ascorbate, full reduction occurred after 24 hours with a 10-fold excess of sodium ascorbate, in line with the reduction potential of each reductant (240 mV for L-glutathione and 140 mV for sodium ascorbate). The dichlorido compound was also rapidly reduced in the presence of as little as 0.1% fetal bovine serum (FBS), with a maximum half life of 5 minutes. We therefore tested both sodium ascorbate, L-glutathione and 10% FBS in phenol red free media; the corresponding Pt^{II}- and Pt^{IV}-salphens were added to cuvettes at a final concentration of 2 µM in either phenol red free buffer with 10% FBS or pH 7.3 PBS buffer. To these cuvettes were added 10 equivalents of c-Myc G4 DNA (20 µM). The fluorescence emission was measured using $\lambda_{\text{ex}} = 375 \text{ nm}$ and $\lambda_{\text{em}} = 500\text{--}700 \text{ nm}$. Subsequently, either L-glutathione (10 mM) or ascorbic acid (10 mM) were added to the PBS and the cuvettes were incubated at 37 °C. At various timepoints the emission was measured (Figure 5.13).

Interestingly, in all cases compound **51** showed no signs of degradation. Although the reduction potential of the dicarboxylate compound is likely to be around -800 mV, significantly higher than both sodium ascorbate and L-glutathione, the in vitro activation of Pt^{IV} prodrugs is complex and also depends heavily on the kinetics of reduction. Furthermore, many studies have shown that the reduction potential is not necessarily linked to how quickly the compounds are reduced in vitro and compounds with hydroxido ligands can be reduced faster than those with carboxylato ligands whilst having a higher reduction potential. This is because the reduction can take place via either an inner or outer sphere process. Indeed, a number of studies have shown that a range of Pt^{IV} prodrugs (including satraplatin) are not reduced at all by some thiols,^[255–257] pointing towards other

mechanisms of intracellular activation and demonstrating the complexity of the reducing environment in cells. In addition to the axial ligands, the equatorial ligands play an important role; both σ -donor strength and steric hindrance can effect the reduction kinetics. The majority of studies have also focussed on single and bi-dentate ligands so less is known about tri- and tetra-dentate systems.

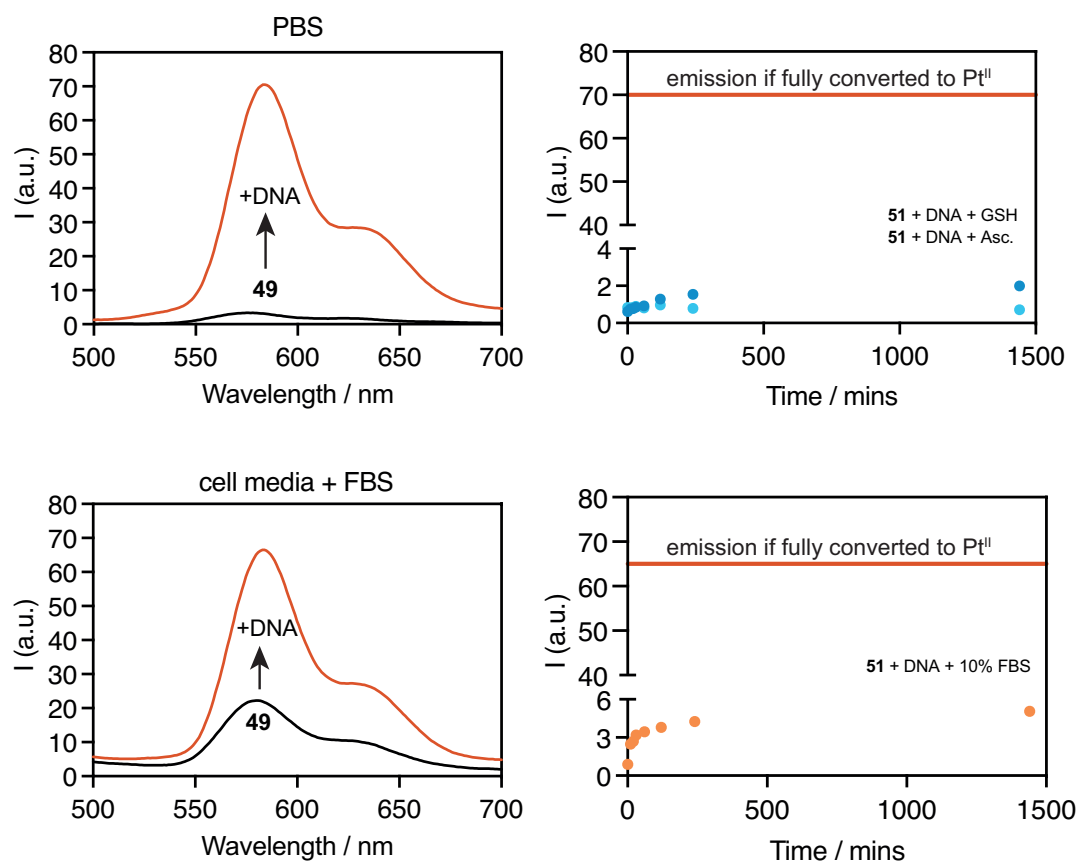


Figure 5.13 Activation and stability experiments with Pt^{IV} -salphen **51** in the presence of various bio-reductants.

We were, however, very pleased to see that the compound was stable in the presence of FBS as this meant we could investigate the cellular properties of the compound. Cell viability experiments were therefore carried out with Pt^{II} -salphen **49** and Pt^{IV} -salphen **51**. Identical conditions to those discussed previously were used, in which U2OS cells were treated with the appropriate compound for 24 hours, after which an MTS assay was used to determine the level of cell growth inhibition. The results are shown in Figure 5.14; as expected Pt^{II} -salphen **49** was shown to be highly cytotoxic with an IC_{50} value of 2 μM . In compari-

son Pt^{IV} -salphen **51** was non-cytotoxic even at the highest concentration tested. There are two potential reasons why this could be the case. Firstly, it might be that the Pt^{IV} -salphens are not cell permeable. However, this seems unlikely given that the Pt^{II} -salphen version is cell permeable and in the group we have shown that other octahedral metal salphens with piperidine side-chains are also cell permeable. The other option is that the compound is internalised, but isn't reduced within 24 hours in U2OS cells. In this case, the compound might require even stronger reducing conditions such as cells grown under an anaerobic environment.

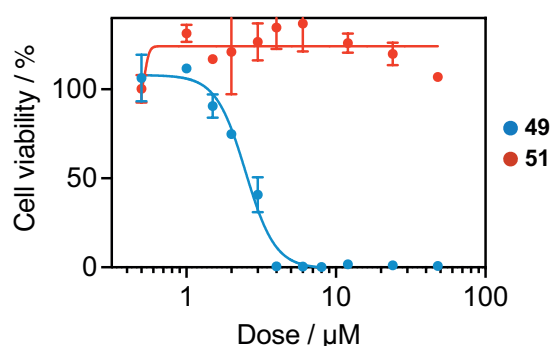


Figure 5.14 Cell viability experiments with U2OS cells (24 hour incubation).

5.5 Conclusions and future work

In conclusion, a new Pt^{IV} -salphen has been synthesised, characterised and tested. The Pt^{IV} -salphen shows vastly decreased interactions with duplex and G4 DNA and offers the much increased stability over the dichlorido analogue that we set out to achieve. However, at this point it is unclear whether these complexes are too stable, and therefore cannot be effectively activated under hypoxic conditions in tumour cells or if this increased stability can be used to our advantage to target particular tumours whilst leaving healthy tissue unharmed.

In order to confirm this behaviour a number of additional experiments will need to be carried out. Firstly, a range of cell lines should be tested, including cells grown under hypoxic conditions. Secondly, ICP-MS should be used to determine the Pt content in cells as the Pt^{IV} -salphen is not emissive so microscopy cannot be used. This should allow us to

build up a clear picture of whether this compound has potential to be used in any further applications. Finally, it might be that we can modify the axial ligands to provide a different mechanism of activation. Some interesting work has been published showing how certain axial ligands can be used to trigger a much faster reduction for example by interacting with ROS or by incorporating a light activated trigger.^[258,259] Both of these options might allow us to utilise the stable nature of the Pt^{IV}-salphen system to create a new family of highly specific Pt^{IV}-prodrugs.

Chapter 6

Conclusions

The aim of this research was to expand the toolkit of methods to investigate and target G4 DNA in cells. This work can broadly be divided into two areas; in the first, we have designed novel compounds which target higher order G4 structures, in particular those which are potentially found at the telomeres. The motivation behind this work was to target the hopefully unique interface at a multimeric G4 structure in order to gain specificity for this type of G4. We firstly established a route to synthesise clickable Ni^{II}- and Pt^{II}-salphens in excellent yield. These complexes were then used to generate a small library of dimeric Ni^{II}-salphens and a larger library of Pt^{II}-salphens with peptide linkers. Via CD melting these complexes showed excellent selectivity for dimeric over monomeric G4 structures with a strong dependence on the linker length separating both the G4 units and the Pt^{II}-salphen units. Fluorescence titrations showed a slightly more complex picture; the selectivity for dimeric over monomeric G4 DNA was less clear and appeared to depend much more on which peptide linker was used. Additionally, binding selectivity was highly dependent on the G4 topology. Unfortunately, the cell permeability was a limiting factor. However, these compounds did show promise and it might be possible to further explore methods to aid cellular internalisation.

Next, a different type of multimeric G4 ligand was investigated, in which one Pt^{II}-salphen unit was tethered to a triangulenium unit. The aim of this was to develop a dual channel probe with a unique signal for monomeric and dimeric DNA. Azide functionalised derivatives of ADOTA-M and DAOTA-M2 (two triangulenium probes which have been previously studied in our group) were designed and synthesised and click with two alkyne functionalised Pt^{II}-salphen derivatives to generate a small library of compounds. Whilst it proved difficult to separate the the two channels into distinct signals, analysis of the emis-

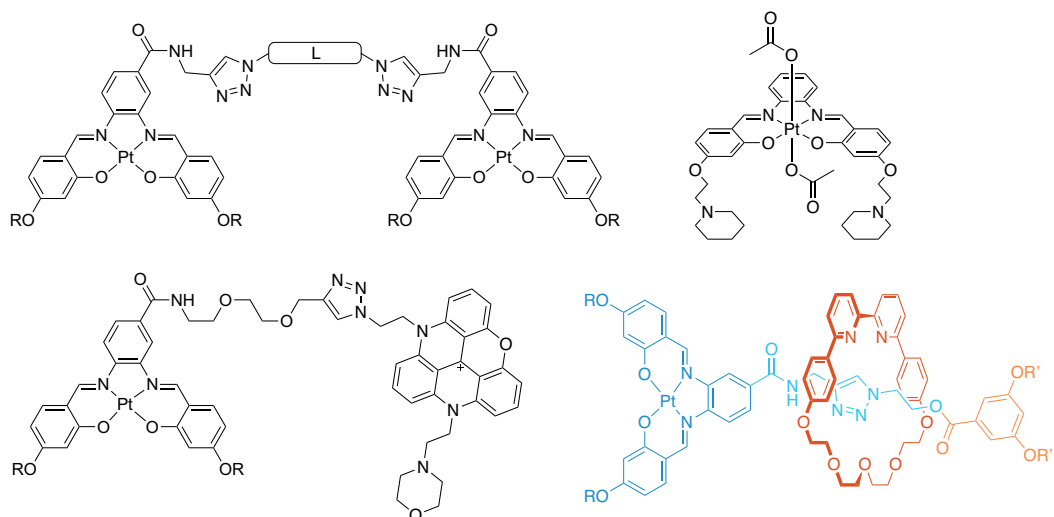


Figure 6.1 A selection of the complexes synthesised in this thesis.

sion lifetime of the probes in the presence of various DNA structures led to some interesting results. In particular, the emission lifetimes of the Pt^{II} -salphen–DAOTA linked probe were around 10 times longer than that of the Pt^{II} -salphen alone. One hypothesis for this is that the probe undergoes triplet-triplet energy transfer upon excitation of the Pt^{II} -salphen moiety, leading to a long-lived triplet localised on the DAOTA core. Importantly, the lifetime of the emission was highly dependent on DNA structure used and highly distinct lifetimes were found for duplex DNA, monomeric G4 DNA and dimeric G4 DNA. Transient absorption spectroscopy experiments will therefore be needed to probe the triplet-triplet energy transfer hypothesis as well as seeing whether there is consistency in emission lifetimes across a range of duplex and G4 DNA structures.

The second theme of the work carried out in this thesis has been looking at how the interactions between G4 DNA-targeting ligands and DNA can be controlled, continuing to focus on Pt^{II} -salphens. In the first case this has involved designing and synthesising a range of interlocked molecules which contain a Pt^{II} -salphen unit caged by a macrocycle. We were able to demonstrate that the macrocycle effectively blocked the ability of the system to bind to both duplex and G4 DNA. Through the inclusion of a modular, cleavable stopper group we provided a mechanism with which to release the Pt^{II} -salphen; we showed that both enzymatically and light-activated release led to liberation of the Pt^{II} -salphen and restoration of binding. Importantly, we were able to switch on the cytotoxicity of the compounds in

cancer cells and show that the cellular target changed after activation.

Finally, the potential of Pt^{II}-salphens to be used as hypoxia activated G4 DNA binders was tested. In this case, the coordination of the Pt centre was switched to an octahedral complex via oxidation, in which the bulky axial ligands block any DNA interactions. Here, we were looking to improve on a first generation compound with chlorido ligands which had limited stability and an unreliable synthesis. Firstly, we devised a synthetic route to obtain a carboxylato Pt^{IV}-salphen; we then demonstrated that this compound could not bind to DNA and was much more stable to reduction than the chlorido analogue. Further work is needed fully characterise this system but it shows promise to further expand the scope of Pt^{IV}-based prodrugs.

In conclusion, we have shown how metal complexes can be used for a wide variety of applications; in particular we have demonstrated how the emissive, fluorescence lifetime and redox properties of Pt^{II}-salphens lead to some very interesting behaviour in a biological context. We hope that this provides further evidence for the necessity of continuing to investigate metal-based complexes for use in medicinal chemistry and imaging.

Chapter 7

Experimental

7.1 General procedures

^1H NMR spectra were recorded on either a Bruker Avance 400 MHz or 500 MHz Ultrashield NMR spectrometer and chemical shifts are reported in parts per million (ppm). Assignments were carried out where possible. Mass spectrometric analysis was performed on a LCT Premier mass spectrophotometer. All chemicals were purchased from Sigma–Aldrich, Fluorochem, Thermo-Scientific or VWR and used without further purification. Flash chromatography was performed using a Teledyne ISCO RF 200 Combiflash system with Redisep Rf Silica Gel flash columns. LCMS was carried out using a Waters ACQUITY UPLC system using a gradient of 5-95% MeCN in water with 0.1% formic acid using a C_{18} column. All compounds used for DNA titrations were dissolved in DMSO to give stock solutions of 5 mM. Stock solutions were diluted to their final concentrations in the appropriate buffers.

7.2 Synthesis

Vilar Policy on Data Collection

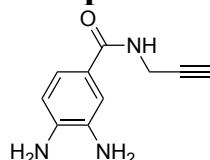
For known compounds (reported in the literature or from other theses), a ^1H NMR spectra is sufficient for use as a synthetic intermediate. For unknown (novel) small molecule compounds a ^1H NMR, ^{13}C NMR and mass measurement is required. For compounds to be used in DNA binding experiments, elemental analysis (or LCMS in the case of insufficient compound) is required. In the subsequent section known compounds are indicated with an asterisk and reasons for any missing data are stated. For bioconjugate compounds containing peptides LCMS analysis is deemed sufficient, in line with other reported com-

plexes.^[161,260]

7.2.1 Chapter 2

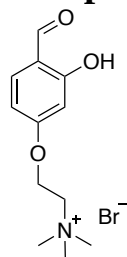
Compounds **22** and **24** were provided by Dr Viktoria Rakers.

Compound 1*



Compound synthesised according to established protocols.^[261] 3,4-Dihydroxybenzoic acid (500 mg, 3.3 mmol) was dissolved in dry DMF (35 mL) to yield a clear, red-brown solution. To this solution, HOBT (666 mg, 4.93 mmol), triethylamine (1.36 mL, 989 mmol) and EDC·HCl (945 mg, 4.93 mmol) were added. After 30 min, propargylamine (368 μ L, 5.75 mmol) was added drop-wise and the reaction stirred for 2 days. The reaction was subsequently quenched with water. The mixture was extracted with EtOAc (3 x 30 mL) and once with DCM (1 x 40 mL). The organic layers were combined and dried with anh. MgSO₄. The solution was filtered under gravity and the solvent removed under reduced pressure. The resultant red-orange oil was purified by column chromatography (silica, 95:5 DCM:methanol, 0.1% triethylamine). The combined fractions were concentrated under reduced pressure to obtain **1** in 32% yield (348 mg, 1.84 mmol). ¹H NMR (400 MHz, MeOD) δ /ppm 7.16 (d, J = 2.1 Hz, 1H), 7.11 (dd, J = 8.2, 2.1 Hz, 1H), 6.65 (d, J = 8.1 Hz, 1H), 4.08 (d, J = 2.6 Hz, 2H), 2.54 (t, J = 2.5 Hz, 1H).

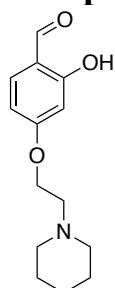
Compound 2*



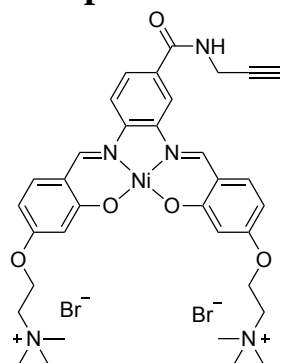
Compound synthesised according to established protocols.^[179] A solution of 2,4-dihydroxybenzaldehyde (10.0 g, 72.4 mmol), 1,2-dibromoethane (6.24 mL, 72.4 mmol)

and potassium carbonate (10.0 g, 72.4 mmol) in acetone (300 mL) was heated at 60 °C for 3 days under nitrogen. The solution was concentrated under reduced pressure and the 4-(2-bromoethoxy)-2-hydroxybenzaldehyde intermediate was purified by column chromatography (Si, 90:10 hexane:EtOAc). This product was then dissolved in 60 mL of an ethanolic solution of trimethylamine. The yellow-brown solution was heated at 50 °C for 12 hours and then concentrated to obtain **2** in a 10% yield (2.20 g, 7.24 mmol). ¹H NMR (400 MHz, MeOD) δ /ppm 10.01 (s, 1H), 7.66 (d, J = 8.7 Hz, 1H), 6.62 (dd, J = 8.7, 2.3 Hz, 1H), 6.53 (d, J = 2.4 Hz, 1H), 4.50 (s, 2H), 3.77 (t, J = 4.6 Hz, 2H), 3.15 (s, 9H).

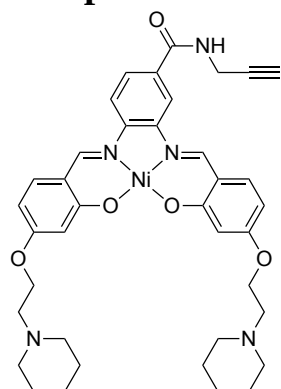
Compound 3*



Compound synthesised according to established protocols.^[114] Dried sodium hydrogencarbonate (3.80 g 36.2 mmol), 1-(2-chloroethyl)piperidine hydrochloride (6.66 g, 36.2 mmol) and 2,4-dihydroxybenzaldehyde (5.00 g, 36.2 mmol) were added to a two-necked round bottom flask, after which the system was flushed with nitrogen. Acetone (250 mL) was added via the side arm, after which the yellow reaction mixture was heated at 60 °C for 3 days. The mixture was filtered, concentrated down, and dried on a high vacuum line to yield a sticky brown solid. The crude mixture was purified via column chromatography (silica, 80:15:10 EtOAc:MeCN:MeOH) to give **3** in 18% yield (1.62 g, 6.51 mmol). ¹H NMR (400 MHz, MeOD) δ /ppm 10.00 (s, 1H), 7.61 (d, J = 8.7 Hz, 1H), 6.56 (dd, J = 8.7, 2.3 Hz, 1H), 6.47 (d, J = 2.3 Hz, 1H), 4.11 (t, J = 5.9 Hz, 2H), 2.64 (t, J = 5.8 Hz, 2H), 2.41 (t, J = 5.2 Hz, 4H), 1.48 (p, J = 5.5 Hz, 4H), 1.37 (td, J = 6.3, 3.1 Hz, 2H).

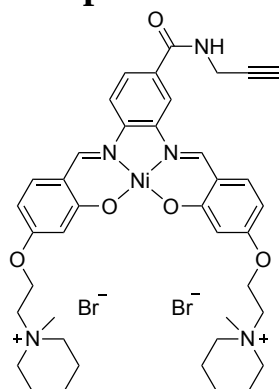
Compound 4*

Compound synthesised according to established protocols.^[261] Compound **1** (50 mg, 0.264 mmol) and compound **2** (169 mg, 0.555 mmol) were dissolved in methanol and stirred for 30 mins at 60 °C to form a bright yellow solution. To the reaction mixture was added $Ni(OAc)_2$ (72.3 mg, 0.980 mmol), with a colour change to red observed. The reaction was stirred for 12 hours at 60 °C before being allowed to cool to room temperature. The solvent was removed under reduced pressure and redissolved in DCM. Precipitation of a red solid was achieved by adding diethyl ether. The solid was further washed with diethyl ether (3 x 10 mL) and dried to afford **4** in an 80% yield (173 mg, 0.211 mmol). ^1H NMR (400 MHz, $DMSO-d_6$) δ /ppm 9.02 (t, J = 5.5 Hz, 1H), 8.82 (d, J = 5.3 Hz, 2H), 8.55 (d, J = 1.7 Hz, 1H), 8.14 (d, J = 8.9 Hz, 1H), 7.76 (dd, J = 8.7, 1.6 Hz, 1H), 7.62 (d, J = 9.0 Hz, 1H), 7.56 (d, J = 8.9 Hz, 1H), 6.47 (d, J = 2.4 Hz, 2H), 6.41 (ddd, J = 8.9, 2.4, 1.3 Hz, 2H), 4.51 (d, J = 5.5 Hz, 4H), 4.12 – 4.10 (m, 2H), 3.81 (t, J = 4.8 Hz, 4H), 3.18 (s, 18H), 3.16 (s, 1H). TOF MS (ES^+) calculated for $[C_{34}H_{41}N_5O_5Ni]^{2+}$ 328.6208, found 328.6230. Elemental analysis (%) $C_{34}H_{41}Br_2N_5NiO_5 \cdot 4H_2O$ found: C 45.87, H 5.55, N 7.87; calculated: C 45.82, H 5.09, N 7.77.

Compound 5

Compound **1** (50 mg, 0.264 mmol) and compound **3** (138 mg, 0.555 mmol) were dissolved in methanol and stirred for 30 mins at 60 °C to form a bright yellow solution. To the reaction mixture was added Ni(OAc)₂ (72.3 mg, 0.980 mmol), with a colour change to red observed. The reaction was stirred for 12 hours at 60 °C before being allowed to cool to room temperature. The solvent was removed under reduced pressure and redissolved in DCM. Precipitation of a red solid was achieved by adding diethyl ether (3 x 10 mL). The solid was further washed with diethyl ether and dried to afford **5** in a 75% yield (140 mg, 0.198 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ/ppm 8.96 (t, J = 5.5 Hz, 1H), 8.71 (d, J = 5.0 Hz, 2H), 8.48 (d, J = 1.7 Hz, 1H), 8.10 (d, J = 8.9 Hz, 1H), 7.71 (dd, J = 8.7, 1.6 Hz, 1H), 7.52 (d, J = 8.9 Hz, 1H), 7.47 (d, J = 8.8 Hz, 1H), 6.41 – 6.30 (m, 4H), 4.16 – 4.04 (m, 6H), 3.17 (t, J = 2.5 Hz, 1H), 2.65 (t, J = 5.8 Hz, 4H), 2.43 (t, J = 5.3 Hz, 8H), 1.50 (p, J = 5.5 Hz, 8H), 1.38 (d, J = 6.1 Hz, 4H). TOF MS (ES⁺) calculated for [C₃₈H₄₃N₅O₅Ni]⁺ 708.2706, found 708.2712.

Compound 6

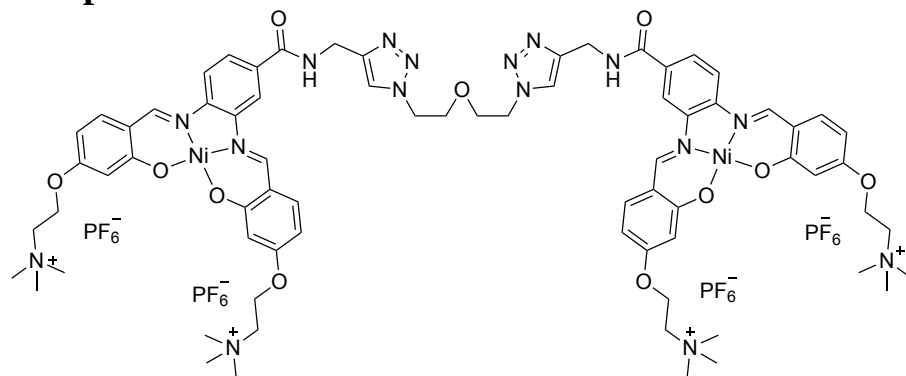


Compound **5** (100 mg, 0.142 mmol) was dissolved in methanol. Methyl iodide (43.1 μL, 1.42 mmol) was added and the reaction stirred for 16 hours. The resulting precipitate was filtered, washed with methanol (3 x 10 mL) and dried under vacuum to afford **6** in 49% yield (69.0 mg, 0.0702 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ/ppm 8.98 (t, J = 5.6 Hz, 1H), 8.81 (d, J = 4.4 Hz, 2H), 8.51 (s, 1H), 8.14 (d, J = 8.8 Hz, 1H), 7.75 (d, J = 8.6 Hz, 1H), 7.61 (d, J = 9.0 Hz, 1H), 7.55 (d, J = 8.9 Hz, 1H), 6.47 (d, J = 2.3 Hz, 2H), 6.41 (d, J = 9.0 Hz, 2H), 4.52 (s, 4H), 4.11 (dd, J = 5.4, 2.6 Hz, 2H), 3.84 (s, 4H), 3.44 (q, J = 5.9 Hz, 8H), 3.18 (t, J = 2.9 Hz, 1H), 3.14 (s, 6H), 1.92 – 1.75 (m, 8H), 1.56 (d, J = 7.0 Hz, 4H). TOF MS (ES⁺) calculated for [C₄₀H₄₉I₂N₅O₅Ni]²⁺ 368.6543, found 368.6547. Elemental analysis (%)

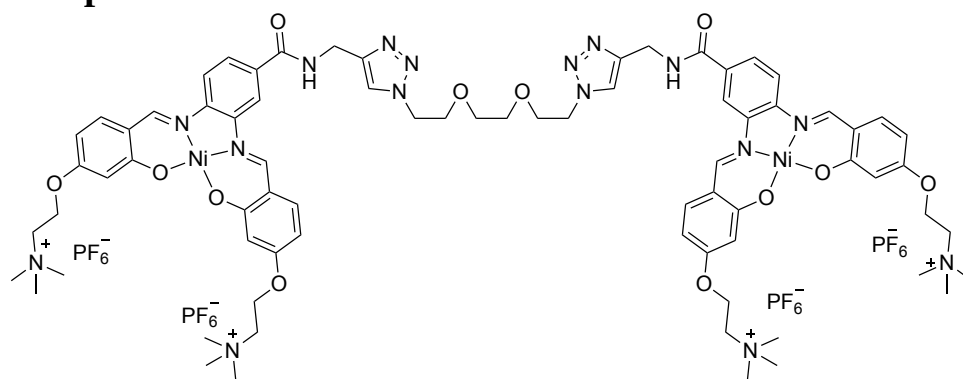
$C_{40}H_{49}I_2N_5NiO_5 \cdot 3H_2O$ found: C 46.31, H 5.25, N 6.75; calculated: C 46.05, H 4.90, N 6.59.

^{13}C NMR not measured at time, no remaining sample.

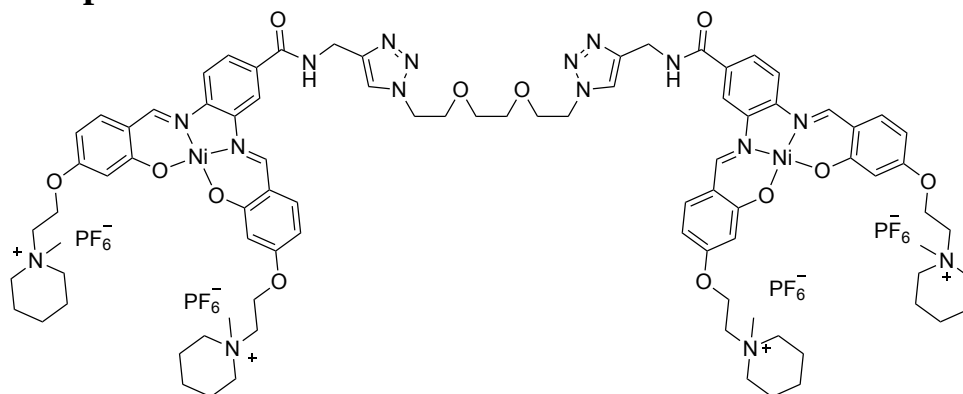
Compound 7



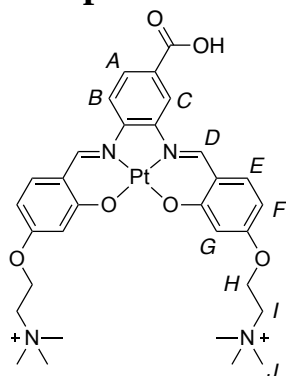
Compound **4** (40.0 mg, 0.0489 mmol) and azido-2-(2-azidoethoxy)ethane (3.66 mg, 0.0235 mmol) were dissolved in water (10 mL). $CuSO_4 \cdot 5H_2O$ (12.2 mg, 0.0489 mmol) and sodium ascorbate (4.65 mg, 0.0489 mmol) were added and the reaction stirred at 60 °C for 16 hours. The solvent was reduced by half via rotary evaporation and the product was precipitated out using methanol. The red solid was redissolved in water and a solution of sat. aq. $NaPF_6$ was added, forming a red suspension which was filtered under reduced pressure. The resulting red solid was washed with water (3 x 5 mL) and dried under vacuum to give **7** in 78% yield (39.1 mg, 0.0191 mmol). ^1H NMR (400 MHz, $DMSO-d_6$) δ /ppm 9.04 (t, J = 5.8 Hz, 2H), 8.73 (d, J = 14.6 Hz, 4H), 8.50 (s, 2H), 8.09 (d, J = 8.9 Hz, 2H), 7.89 (s, 2H), 7.82 – 7.73 (m, 2H), 7.53 (t, J = 8.9 Hz, 4H), 6.46 (d, J = 2.4 Hz, 4H), 6.40 (dt, J = 8.8, 2.3 Hz, 4H), 4.53 (d, J = 5.5 Hz, 4H), 4.49 (m, 14H), 3.80 (m, 14H), 3.17 (s, 36H). Elemental analysis (%) $C_{72}H_{88}F_{24}N_{16}Ni_2O_{11}P_4 \cdot 5H_2O$ found: C 44.01, H 5.15, N 9.55; calculated: C 43.62, H 4.97, N 9.61. ^{13}C NMR not measured at the time, no remaining sample.

Compound 8

Compound **4** (40.0 mg, 0.0489 mmol) and bis(2-azidoethoxy)ethane (4.70 mg, 0.0235 mmol) were dissolved in water (10 mL). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (12.2 mg, 0.0489 mmol) and sodium ascorbate (4.65 mg, 0.0489 mmol) were added and the reaction stirred at 60 °C for 16 hours. The solvent was reduced by half via rotary evaporation and the product was precipitated out using methanol. The red solid was redissolved in water and a solution of sat. aq. NaPF_6 was added, forming a red suspension which was filtered under reduced pressure. The resulting red solid was washed with water (3 x 20 mL) and dried under vacuum to give **8** in 85% yield (39 mg, 0.0190 mmol). ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 9.07 (s, 2H), 8.77 (s, 2H), 8.74 (s, 2H), 8.50 (s, 2H), 8.10 (d, $J = 8.9$ Hz, 2H), 7.96 (s, 2H), 7.80 – 7.73 (m, 2H), 7.57 (d, $J = 9.0$ Hz, 2H), 7.53 (d, $J = 9.0$ Hz, 2H), 6.46 (d, $J = 2.4$ Hz, 4H), 6.40 (dt, $J = 8.8, 2.5$ Hz, 4H), 4.54 (d, $J = 4.8$ Hz, 4H), 4.52 – 4.44 (m, 12H), 3.84 – 3.70 (m, 12H), 3.48 (s, 4H), 3.17 (s, 36H). TOF MS (ES^+) calculated for $[\text{C}_{74}\text{H}_{92}\text{N}_{16}\text{Ni}_2\text{O}_{12}]^{4+}$ 379.1484, found 379.1445. Elemental analysis (%) $\text{C}_{74}\text{H}_{92}\text{F}_{24}\text{N}_{16}\text{Ni}_2\text{O}_{12}\text{P}_4 \cdot 4\text{H}_2\text{O} \cdot 2\text{NaPF}_6$ found: C 35.62, H 4.28, N 8.98; calculated: C 35.60, H 4.02, N 8.65. ^{13}C NMR not measured at the time, no remaining sample.

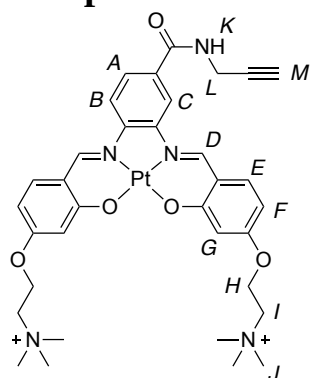
Compound 10

Compound **5** (48.5 mg, 0.0489 mmol) and bis(2-azidoethoxy)ethane (4.70 mg, 0.0235 mmol) were dissolved in water (10 mL). $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (12.2 mg, 0.0489 mmol) and sodium ascorbate (4.65 mg, 0.0489 mmol) were added and the reaction stirred at 60 °C for 16 hours. The solvent was reduced by half via rotary evaporation and the product was precipitated out using methanol. The red solid was redissolved in water and a solution of sat. aq. NaPF_6 was added, forming a red suspension which was filtered under reduced pressure. The resulting red solid was washed with water (3 x 20 mL) and dried under vacuum to give **10** in 80% yield (40.1 mg, 0.0147 mmol). ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 9.04 (t, J = 5.8 Hz, 2H), 8.73 (d, J = 14.6 Hz, 4H), 8.50 (s, 2H), 8.09 (d, J = 8.9 Hz, 2H), 7.89 (s, 2H), 7.82 – 7.73 (m, 2H), 7.53 (t, J = 8.9 Hz, 4H), 6.46 (d, J = 2.4 Hz, 4H), 6.40 (dt, J = 8.8, 2.3 Hz, 4H), 4.53 (d, J = 5.5 Hz, 4H), 4.49 (d, J = 5.2 Hz, 12H), 3.80 (q, J = 4.9 Hz, 12H), 3.17 (s, 36H). TOF MS (ES^+) calculated for $[\text{C}_{86}\text{H}_{108}\text{N}_{16}\text{Ni}_2\text{O}_{12}]^{4+}$ 419.1798, found 419.1795. Elemental analysis (%) $\text{C}_{86}\text{H}_{108}\text{F}_{24}\text{N}_{16}\text{Ni}_2\text{O}_{12}\text{P}_4 \cdot 4\text{H}_2\text{O} \cdot \text{NaPF}_6$ found: C 37.72, H 4.31, N 9.77; calculated: C 37.80, H 4.20, N 9.21. ^{13}C NMR was not measured at the time, no remaining sample.

Compound 13

Compound **2** (630 mg, 2.07 mmol), 3,4-diaminobenzoic acid (150 mg, 0.986 mmol), and $\text{Zn}(\text{OAc})_2 \cdot 2\text{H}_2\text{O}$ (238 mg, 1.08 mmol) were dissolved in DMSO (5 mL). The reaction was stirred at 80 °C for 2 hours before PtCl_2 (288 mg, 1.08 mmol) was added. The reaction mixture was stirred at 80 °C for 3 days before being allowed to cool to room temperature. Methanol (150 mL) was added and the resulting orange precipitate was filtered and washed with methanol (50 mL) and DCM (50 mL) to afford **13** in 82% yield as an orange solid (742 mg, 0.808 mmol). ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 9.37 – 9.32 (m, 2H, D), 8.78 (s, 1H, C), 8.39 – 8.33 (m, 1H, B), 7.88 (d, J = 8.8 Hz, 2H, F), 7.76 (d, J = 9.1 Hz, 1H, A), 6.63 (s, 2H, G), 6.49 (t, J = 6.7 Hz, 2H, E), 4.56 (s, 4H, H), 3.87 – 3.77 (m, 4H, I), 3.20 (s, 18H, J). ^{13}C NMR (101 MHz, DMSO-d_6) δ /ppm 166.54, 166.30, 163.56, 163.37, 150.19, 144.02, 137.35, 137.09, 128.04, 117.17, 108.16, 107.74, 103.17, 63.89, 61.73, 53.16, 48.62. TOF MS (ES^+) calculated for $[\text{C}_{31}\text{H}_{38}\text{N}_4\text{O}_6\text{Pt}]^{2+}$ 378.6221, found 378.6215. HPLC-MS(ESI) calculated for $[\text{M}]^{2+}$ 378.6, found 378.6.

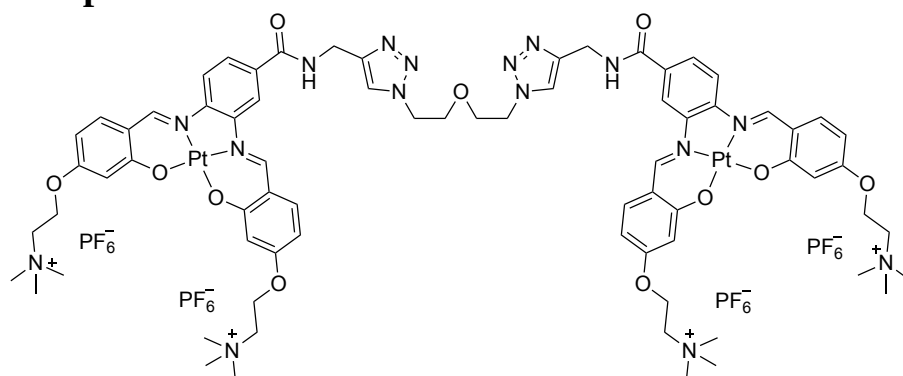
Compound 15



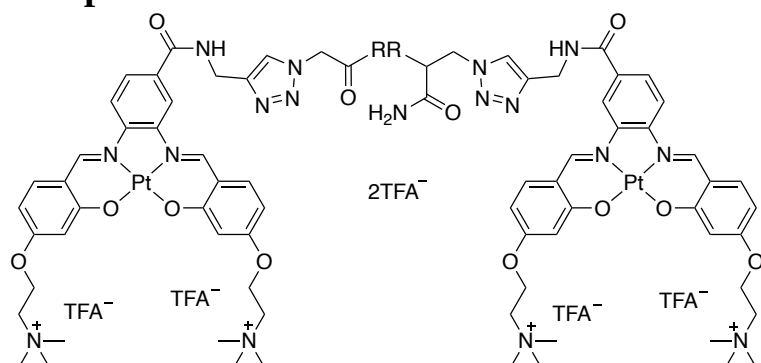
Compound **13** (500 mg, 0.603 mmol), HOBT (123 mg, 0.905 mmol), EDC·HCl (174 mg, 0.905 mmol) and triethylamine (126 μL , 0.905 mmol) were dissolved in DMSO (5 mL). The reaction mixture was stirred under nitrogen for 30 mins before propargyl amine (67.6 μL , 0.905 mmol) was added. The reaction was stirred at 45 °C for 12 hours. The reaction mixture was diluted with water (100 mL) and a solution of sat. aq. NaPF_6 (15 mL) was added. The resulting orange precipitate was filtered and washed with water (20 mL) and dried under vacuum to obtain **15** in 95% yield as an orange solid (622 mg, 0.573 mmol). ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 9.40 (m, 2H, D), 9.07 (t, J = 5.5 Hz, 1H, K), 8.80 (s, 1H, C), 8.44 (d,

J = 9.1 Hz, 1H, B), 7.90 – 7.83 (m, 2H, F), 7.80 (d, J = 9.1 Hz, 1H, A), 6.70 (s, 2H, G), 6.59 – 6.47 (m, 2H, E), 4.57 (t, J = 4.8 Hz, 4H, H), 4.14 (dd, J = 5.5, 2.5 Hz, 2H, L), 3.82 (t, J = 4.8 Hz, 4H, I), 3.19 (s, 18H, J), 3.16 (s, 1H, M). ^{13}C NMR (101 MHz, DMSO- d_6) δ /ppm 166.67, 166.48, 164.80, 163.72, 163.55, 150.70, 150.32, 144.15, 137.17, 132.28, 125.78, 117.18, 117.06, 116.05, 115.75, 108.33, 108.05, 103.27, 81.11, 73.25, 63.89, 61.73, 53.14, 30.69, 28.73. TOF MS (ES^+) calculated for $[\text{C}_{34}\text{H}_{41}\text{N}_5\text{O}_5\text{Pt}]^{2+}$ 397.1379, found 397.1386. HPLC-MS(ESI) calculated for $[\text{M}]^{2+}$ 397.1, found 397.1.

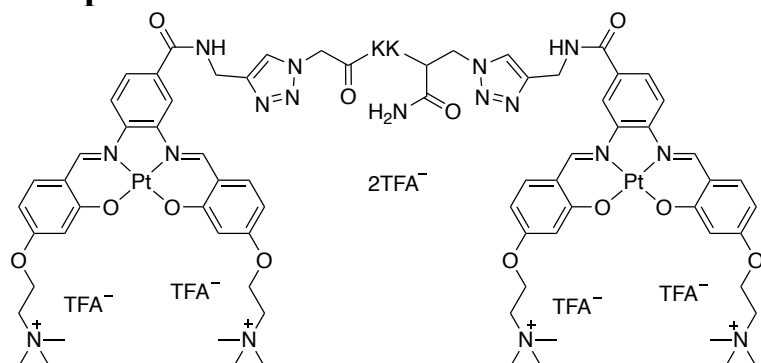
Compound 16



Compound **15** (20 mg, 0.0184 mmol) and azido-2-(2-azidoethoxy)ethane (1.38 mg, 0.00885 mmol) were added to DMSO, followed by the addition of $\text{Cu}(\text{CH}_3\text{CN})_4 \cdot \text{PF}_6$ (6.87 mg, 0.0184 mmol). The reaction was stirred at rt for 16 hours before precipitation with DCM. The solid was centrifuged and washed a further 3 times with DCM and subsequently purified via semi-preparative HPLC to afford **16** in 46% yield (9.62 mg, 0.00846 mmol). ^1H NMR (400 MHz, DMSO- d_6) δ /ppm 9.31 (d, J = 15.5 Hz, 3H), 9.12 (t, J = 5.6 Hz, 2H), 8.77 (d, J = 1.7 Hz, 2H), 8.37 (d, J = 8.9 Hz, 2H), 7.90 (s, 2H), 7.89 – 7.81 (m, 2H), 7.77 (dd, J = 9.2, 7.7 Hz, 3H), 6.67 (d, J = 2.4 Hz, 4H), 6.51 (dd, J = 9.0, 2.4 Hz, 4H), 4.55 (t, J = 6.9 Hz, 13H), 4.49 (t, J = 5.1 Hz, 4H), 3.81 (q, J = 5.5, 4.2 Hz, 13H), 3.19 (s, 41H). ^{13}C NMR (400 MHz, DMSO- d_6) δ /ppm 206.53, 166.60, 163.64, 163.48, 150.52, 150.07, 146.34, 137.08, 125.84, 123.47, 117.15, 115.92, 115.54, 108.24, 107.98, 103.23, 68.48, 63.89, 61.70, 54.90, 53.16, 49.17, 40.18, 39.97, 34.92, 30.70. HPLC-MS(ESI) calculated for $[\text{M}]^{4+}$ 436.2, found 436.5.

Compound 17

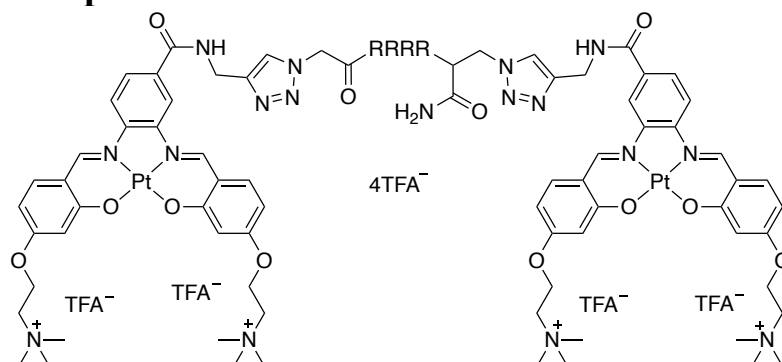
Compound **15** (20 mg, 0.0184 mmol) was dissolved in DMSO (0.2 mL), peptide **a** (6.66 mg, 0.00885 mmol) was dissolved in water (0.2 mL) and the two were combined, followed by the addition of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4.60 mg, 0.184 mmol) and sodium ascorbate (3.65 mg, 0.0184 mmol). The reaction was stirred for 24 hours before being diluted with 10 mL water and purified via semi-preparative HPLC to afford compound **17** in 45% yield (11.6 mg, 0.00414 mmol). HPLC-MS(ESI) calculated for $[\text{M}]^{4+}$ 528.21, found 528.7; $[\text{M}+\text{HTFA}]^{4+}$ 556.71, found 557.2; $[\text{M}+2\text{HTFA}]^{4+}$ 585.21, found 585.4; $[\text{M}+\text{TFA}]^{3+}$ 742.27, found 742.0; $[\text{M}+\text{TFA}+\text{HTFA}]^{3+}$ 780.27, found 780.35; $[\text{M}+\text{TFA}+2\text{HTFA}]^{3+}$ 818.27, found 818.10; $[\text{M}+2\text{TFA}+\text{HTFA}]^{2+}$ 1227.41, found 1227.20; $[\text{M}+2\text{TFA}+2\text{HTFA}]^{2+}$ 1284.41, found 1283.55.

Compound 18

Compound **15** (20 mg, 0.0184 mmol) was dissolved in DMSO (0.2 mL), peptide **b** (6.16 mg, 0.00885 mmol) was dissolved in water (0.2 mL) and the two were combined, followed by the addition of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4.60 mg, 0.184 mmol) and sodium ascorbate (3.65 mg, 0.0184 mmol). The reaction was stirred for 24 hours before being diluted with 10 mL water and purified via semi-preparative HPLC to afford compound **18** in 35% yield (8.82 mg, 0.00332 mmol). HPLC-MS(ESI) calculated for $[\text{M}]^{4+}$ 514.2, found 514.55; $[\text{M}+\text{TFA}]^{3+}$ 723.6, found

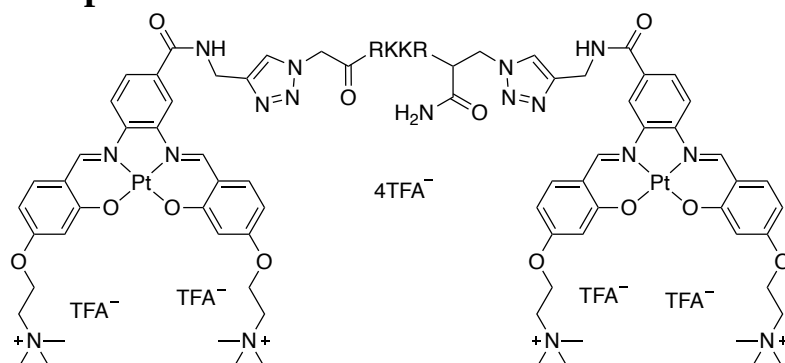
723.5; $[M+2TFA]^{2+}$ 1142.41, found 1141.9.

Compound 19



Compound **15** (20 mg, 0.0184 mmol) was dissolved in DMSO (0.2 mL), peptide **c** (9.42 mg, 0.00885 mmol) was dissolved in water (0.2 mL) and the two were combined, followed by the addition of $CuSO_4 \cdot 5H_2O$ (4.60 mg, 0.184 mmol) and sodium ascorbate (3.65 mg, 0.0184 mmol). The reaction was stirred for 24 hours before being diluted with 10 mL water and purified via semi-preparative HPLC to afford compound **19** in 31% yield (9.51 mg, 0.00285 mmol). HPLC-MS(ESI) calculated for $[M+H]^{5+}$ 485.2, found 485.8; $[M]^{4+}$ 663.26, found 663.45; $[M+HTFA]^{4+}$ 691.76, found 692.2; $[M+2HTFA]^{4+}$ 720.26, found 720.45; $[M+TFA+2HTFA]^{3+}$ 922.34, found 921.9; $[M+TFA+3HTFA]^{3+}$ 960.34, found 955.9; $[M+TFA+4HTFA]^{3+}$ 998.34, found 998.54 $[M+2TFA+3HTFA]^{2+}$ 1497.51, found 1497.5; $[M+2TFA+4HTFA]^{2+}$ 1554.51, found 1554.1.

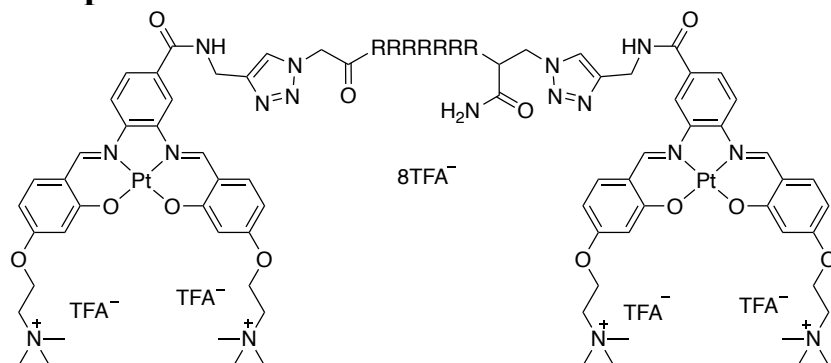
Compound 20



Compound **15** (20 mg, 0.0184 mmol) was dissolved in DMSO (0.2 mL), peptide **d** (8.93 mg, 0.00885 mmol) was dissolved in water (0.2 mL) and the two were combined, followed by the addition of $CuSO_4 \cdot 5H_2O$ (4.60 mg, 0.184 mmol) and sodium ascorbate (3.65 mg, 0.0184 mmol). The reaction was stirred for 24 hours before being diluted with 10

mL water and purified via semi-preparative HPLC to afford compound **20** in 32% yield (9.65 mg, 0.00294 mmol). HPLC-MS(ESI) calculated for $[M+H]^{5+}$ 474.0, found 474.25; $[M]^{4+}$ 592.25, found 593.15; $[M+HTFA]^{4+}$ 620.75, found 620.75; $[M+2HTFA]^{4+}$ 649.25, found 649.65; $[M+TFA+HTFA]^{3+}$ 865.47, found 865.55; $[M+2TFA+HTFA]^{2+}$ 1355.51, found 1355.0; $[M+2TFA+2HTFA]^{2+}$ 1412.51, found 1411.70.

Compound 21



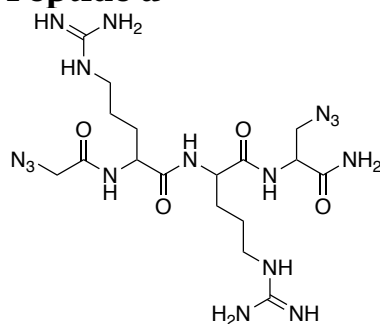
Compound **15** (20 mg, 0.0184 mmol) was dissolved in DMSO (0.2 mL), peptide **e** (15.0 mg, 0.00885 mmol) was dissolved in water (0.2 mL) and the two were combined, followed by the addition of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4.60 mg, 0.184 mmol) and sodium ascorbate (3.65 mg, 0.0184 mmol). The reaction was stirred for 24 hours before being diluted with 10 mL water and purified via semi-preparative HPLC to afford compound **21** in 30% yield (12.2 mg, 0.00276 mmol). HPLC-MS(ESI) calculated for $[M+H]^{5+}$ 508.57, found 508.80; $[M+2HTFA]^{4+}$ 655.69, found 656.05; $[M+3TFA]^{4+}$ 678.49, found 678.60; $[M+4HTFA]^{4+}$ 701.29, found 701.50; $[M+5HTFA]^{4+}$ 724.09, found 724.20; $[M+6HTFA]^{4+}$ 769.69, found 769.75; $[M+TFA+HTFA]^{3+}$ 905.11, found 905.40; $[M+TFA+HTFA]^{3+}$ 933.61, found 934.60; $[M+TFA+HTFA]^{3+}$ 962.11, found 962.90; $[M+TFA+HTFA]^{3+}$ 990.61, found 990.35; $[M+2TFA+HTFA]^{2+}$ 1244.81, found 1244.2; $[M+2TFA+2HTFA]^{2+}$ 1282.81, found 1282.85. $[M+2TFA+2HTFA]^{2+}$ 1320.81, found 1321.400.

Peptide synthesis

All peptide synthesis reagents were purchased from Sigma Aldrich. C-terminal amide peptide was synthesized following standard Fmoc-peptide synthesis protocols on a 0.1 mmol scale using a 0.5 mmol/g loading H-Rink amide ChemMatrix resin (35–100 mesh) from

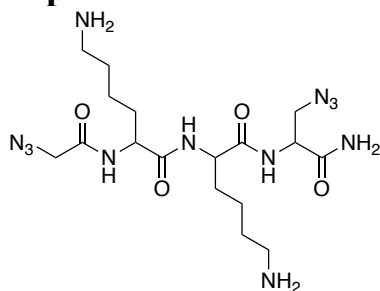
Sigma Aldrich with a Liberty Lite automatic microwave assisted peptide synthesizer from CEM Corporation. The amino acids were coupled in 5-fold excess using oxyme as an activating agent. Couplings were conducted for 4 min at 90 °C. Deprotection of the temporal Fmoc protecting group was performed by treating the resin with 20% piperidine in DMF for 1 min at 75°C. Cleavage and deprotection of the peptide were simultaneously performed using standard conditions by incubating the resin for 2.5 h with an acidic mixture containing 50 μ L DCM, 25 μ L of H₂O, 25 μ L of TIS (triisopropylsilane), and 900 TFA μ L per 40 mg of resin. The resin was filtered, and the TFA filtrate was concentrated under a nitrogen stream to an approximate volume of 1 mL, and then added onto ice-cold diethyl ether (20 mL). After 10–30 min, the precipitate was centrifuged and washed again with 5 mL of ice-cold ether. The solid residue was dried under argon and re-dissolved in acetonitrile/water 1:1 (2–5 mL) and purified by preparative RP-HPLC. Yields were calculated relative to 0.05 mmol resin loading and identity of peptides confirmed via LCMS.

Peptide a



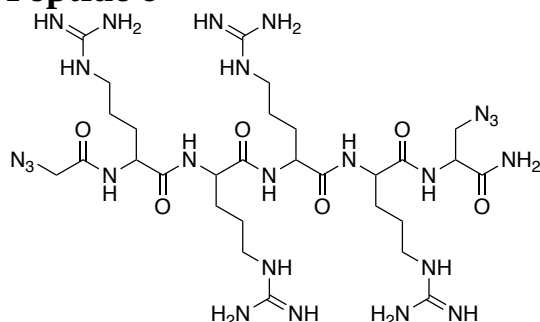
Peptide **a** (41.0 mg, 29 % yield) was synthesised according to the above SPPS protocol. HPLC-MS(ESI) calculated for [M+2H]²⁺ 263.14, found 263.25; [M+H]⁺ 525.28, found 525.35.

Peptide b



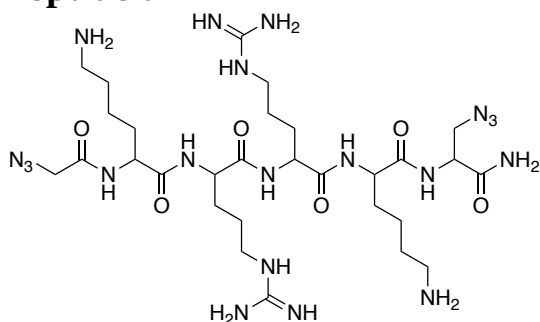
Peptide **b** (37.1 mg, 27 % yield) was synthesised according to the above SPPS protocol. HPLC-MS(ESI) calculated for $[M+2H]^{2+}$ 235.14, found 235.20; $[M+H]^+$ 469.27, found 469.30.

Peptide c



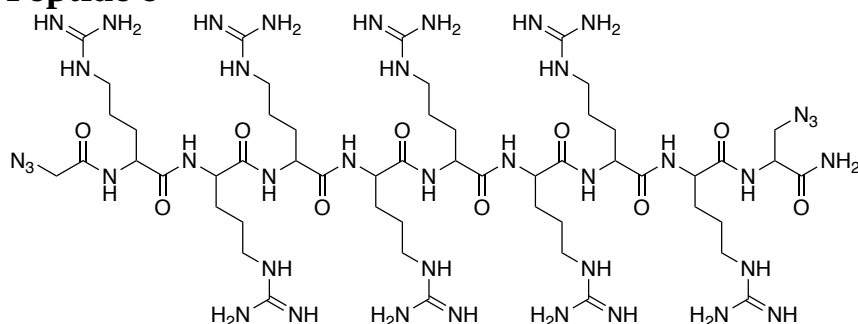
Peptide **c** (29.5 mg, 18 % yield) was synthesised according to the above SPPS protocol. HPLC-MS(ESI) calculated for $[M+3H]^{3+}$ 263.14, found 263.25; $[M+2H]^{2+}$ 525.28, found 525.35; $[M+2H+HTFA]^{2+}$ 476.24, found 476.3; $[M+H]^+$ 837.48, found 837.50.

Peptide d



Peptide **d** (25.1 mg, 15 % yield) was synthesised according to the above SPPS protocol. HPLC-MS(ESI) calculated for $[M+2H]^{2+}$ 391.23, found 391.35; $[M+H]^+$ 781.47, found 781.50.

Peptide e

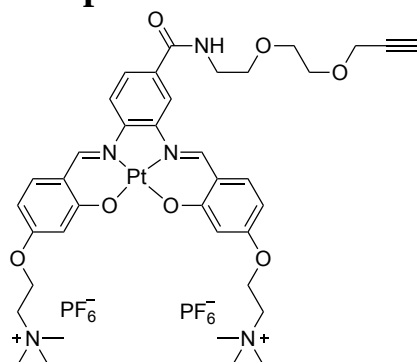


Peptide **e** (28.9 mg, 13 % yield) was synthesised according to the above SPPS protocol. HPLC-MS(ESI) calculated for $[M+3H]^{3+}$ 487.96, found 488.15; $[M+3H+HTFA]^{3+}$ 525.96, found 526.20; $[M+3H+2HTFA]^{3+}$ 563.96, found 564.2; $[M+3H+3HTFA]^{3+}$ 601.96, found 602.2;

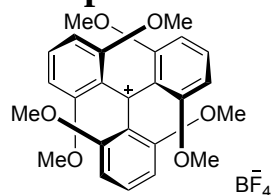
$[M+2H]^{2+}$ 788.45, found 788.45; $[M+2H+HTFA]^{2+}$ 845.45, found 845.70; $[M+2H+2HTFA]^{2+}$ 902.45, found 902.55; $[M+2H+3HTFA]^{2+}$ 959.45, found 959.75.

7.2.2 Chapter 3

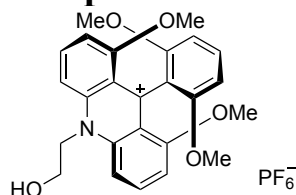
Compound 25



Compound **13** (100 mg, 0.109 mmol), HOBt (22.1 mg, 0.163 mmol), EDC·HCl (31.3 mg, 0.163 mmol) and triethylamine (45.3 μ L, 0.326 mmol) were dissolved in DMSO (2 mL). The reaction mixture was stirred under nitrogen for 30 mins before 2-(2-(prop-2-yn-1-yloxy)ethoxy)ethan-1-amine (39.0 μ L, 0.191 mmol) was added. The reaction was stirred at 45 °C for 12 hours. The reaction mixture was diluted with water (30 mL) and a solution of sat. aq. NaPF₆ (5 mL) was added. The resulting orange precipitate was filtered and washed with water (20 mL) and dried under vacuum to obtain **25** in 95% yield as an orange solid (117 mg, 0.114 mmol). ¹H NMR (400 MHz, DMSO-d₆) δ /ppm 9.40 (d, J = 5.0 Hz, 2H), 8.79 (d, J = 1.7 Hz, 1H), 8.70 (t, J = 5.6 Hz, 1H), 8.43 (d, J = 8.9 Hz, 1H), 7.91 – 7.83 (m, 2H), 7.80 (d, J = 9.1 Hz, 1H), 6.70 (t, J = 2.2 Hz, 2H), 6.54 (dt, J = 8.9, 2.0 Hz, 2H), 4.56 (t, J = 4.5 Hz, 5H), 4.14 (d, J = 2.4 Hz, 2H), 3.82 (t, J = 4.7 Hz, 5H), 3.62 – 3.54 (m, 2H), 3.58 (s, 5H), 3.50 (t, J = 5.5 Hz, 2H), 3.40 (t, J = 2.4 Hz, 3H), 3.19 (s, 23H), 3.16 (s, 1H). ¹³C NMR (101 MHz, DMSO-d₆) δ /ppm 166.63, 165.11, 163.70, 163.54, 150.63, 150.29, 146.28, 144.14, 137.13, 132.90, 125.74, 117.18, 115.95, 115.63, 108.28, 108.03, 103.28, 80.32, 77.16, 69.40, 68.98, 68.50, 63.89, 61.73, 57.52, 53.14, 40.18, 30.70. TOF MS (ES⁺) calculated for [C₃₈H₄₉N₅O₇Pt]⁺ 881.3259, found 881.3234.

Compound 26*

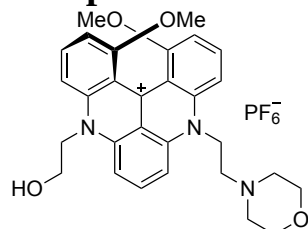
Compound synthesised according to established protocols.^[262] Tetramethylethylenediamine (100 μ L, 0.667 mmol) was added to a solution of n-butyllithium in cyclohexane (2 M) (12 mL, 24 mmol) under a nitrogen atmosphere. The mixture was then cooled to 0 °C before the addition of 1,3-dimethoxybenzene (3.0 mL, 22.9 mmol). After warming to room temperature, dry diethyl ether (10 mL) was added to the white suspension followed by anh. diethyl carbonate (0.900 mL, 7.34 mmol) and the reaction was stirred for 24 hours. Water (50 mL) and ethyl acetate (50 mL) was added and the organic phase collected, followed by washing of the organic phase with more water (2 x 50 mL) before being dried over magnesium sulphate, filtered and concentrated in vacuo to yield an orange oil/solid. This mixture was then suspended in diethyl ether (30 mL) before tetrafluoroboric acid in diethyl ether (1.90 mL, 13.8 mmol) was added. Filtration of the violet suspension and washing with diethyl ether gave a black powder which was used without further purification (3.06 g, 5.99 mmol). ¹H NMR (400 MHz, Chloroform-d) δ /ppm 7.60 (t, J = 8.5 Hz, 3H), 6.53 (d, J = 8.5 Hz, 6H), 3.60 (s, 18H).

Compound 27

Ethanolamine (533 μ L, 8.82 mmol) was added to a solution of **26** (3000 mg, 5.87 mmol) in 1-methyl-2-pyrrolidinone (NMP) (5 mL). The deep orange/red solution formed was left to stir at room temperature for 2 hours before a solution of sat. aq. NaPF₆ (5 mL) and water (50 mL) was added. The red precipitate formed was recovered by filtration and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL) to give compound **27** in 75% yield (2.46 g, 4.41 mmol). ¹H NMR (400 MHz, Acetonitrile-d₃) δ /ppm 8.21 (t, J = 8.6 Hz, 2H), 8.07

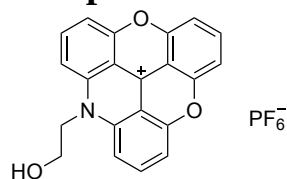
(d, $J = 9.2$ Hz, 2H), 7.47 (t, $J = 8.3$ Hz, 1H), 7.12 (d, $J = 7.9$ Hz, 2H), 6.81 (d, $J = 8.4$ Hz, 2H), 5.32 (t, $J = 5.6$ Hz, 2H), 4.34 (t, $J = 5.6$ Hz, 2H), 3.60 – 3.53 (m, 12H). *Missing accurate mass measurement, not measured at time and no remaining sample.*

Compound 28



4-(2-Aminoethyl)morpholine (3.71 mL, 28.3 mmol) was added to a solution of **27** (1.60 g, 2.83 mmol) in NMP. The reaction mixture was heated to 110 °C for 2 hours, before being cooled to room temperature. The green precipitate formed was recovered by filtration and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL). The product was purified via column chromatography (silica, 50:50 DCM:MeCN, 20 mM NaPF₆) to give compound **28** in 53% yield (878 mg, 1.50 mmol). ¹H NMR (400 MHz, Chloroform-d) δ /ppm 8.20 (t, $J = 8.5$ Hz, 1H), 7.89 (q, $J = 8.4$ Hz, 2H), 7.80 (d, $J = 8.6$ Hz, 1H), 7.59 (d, $J = 8.9$ Hz, 1H), 7.55 (d, $J = 8.5$ Hz, 1H), 7.41 (d, $J = 8.8$ Hz, 1H), 6.83 (dd, $J = 8.0, 1.7$ Hz, 2H), 4.80 (dddt, $J = 76.1, 54.2, 14.9, 6.7$ Hz, 4H), 4.34 (t, $J = 6.4$ Hz, 2H), 3.76 (m, 6H), 3.02 (s, 2H), 2.67 (s, 4H), 1.65 (s, 4H). *Missing accurate mass measurement, not measured at time and no remaining sample.*

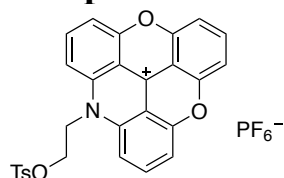
Compound 29



Pyridine·HCl (5.00 g) was added to a round bottom flask and heated to 200 °C for 30 mins. Next, compound **27** (400 mg, 0.710 mmol) was added and the reaction stirred for 1 hour before being cooled to room temperature. The reaction mixture was filtered and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL). The product was purified via column chromatography (silica, 50:50 DCM:MeCN, 20 mM NaPF₆) to give compound **29** in 44 % yield (147 mg, 0.311 mmol). ¹H NMR (400 MHz, Acetonitrile-d₃) δ /ppm 8.34 (dd, $J = 9.0$,

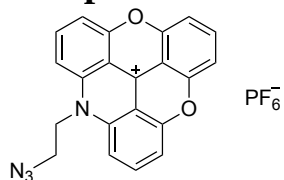
8.2 Hz, 2H), 8.13 (t, $J = 8.5$ Hz, 1H), 7.94 (d, $J = 9.0$ Hz, 2H), 7.57 (dd, $J = 8.2, 0.5$ Hz, 2H), 7.50 (d, $J = 8.5$ Hz, 2H), 4.94 (t, $J = 5.4$ Hz, 2H), 4.18 (q, $J = 5.5$ Hz, 2H), 3.26 (t, $J = 5.6$ Hz, 1H). *Missing ^{13}C NMR, accurate mass measurement, not measured at time and no remaining sample.*

Compound 30



A solution of **29** (50.0 mg, 0.101 mmol) in DCM (20 mL) was cooled to 0 °C followed by the addition of triethylamine (59.8 μL , 0.432 mmol) and tosyl anhydride (70.4 mg, 0.216 mmol). The mixture was stirred for 4 hours, before being diluted with water (20 mL) and extracted with DCM (3 x 20 mL). The organic layer was dried over magnesium sulphate and the solvent removed. The product was then redissolved in acetonitrile and precipitated out with a sat. aq. solution of NaPF₆. The precipitate was washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL) before being dried under reduced pressure to afford **30** in quantitative yield. ^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm 8.31 (dd, $J = 9.0, 8.2$ Hz, 2H), 8.20 (t, $J = 8.5$ Hz, 1H), 7.72 (d, $J = 8.9$ Hz, 2H), 7.62 – 7.54 (m, 4H), 7.22 – 7.05 (m, 2H), 6.83 – 6.76 (m, 2H), 5.02 (t, $J = 5.0$ Hz, 2H), 4.75 (dd, $J = 5.5, 4.6$ Hz, 2H), 2.01 – 1.88 (m, 2H). *Missing ^{13}C NMR, accurate mass measurement, not measured at time and no remaining sample.*

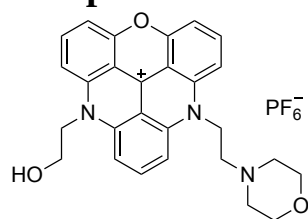
Compound 31



Compound **30** (30 mg, 0.048 mmol) and sodium azide (9.32 mg, 0.143 mmol) were combined in DMSO (0.5 mL) and stirred for 48 hours. Subsequent addition of a sat. aq. solution of NaPF₆ (5 mL) caused the compound to precipitate, after which it was filtered and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL) before being dried under reduced pressure. The product was purified via column chromatography (silica, 50:50

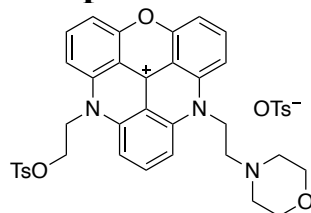
DCM:MeCN, 20 mM NaPF₆) to afford **31** in 94% yield (22.4 mg, 0.045 mmol). ¹H NMR (400 MHz, Acetonitrile-d₃) δ /ppm 8.36 (dd, J = 9.0, 8.2 Hz, 2H), 8.15 (t, J = 8.5 Hz, 1H), 7.89 (d, J = 8.9 Hz, 2H), 7.59 (dd, J = 8.3, 0.5 Hz, 2H), 7.52 (d, J = 8.5 Hz, 2H), 4.95 (t, J = 5.9 Hz, 2H), 4.06 (t, J = 5.9 Hz, 2H). HPLC-MS(ESI) calculated for [M]⁺ 353.1, found 353.1. TOF MS (ES⁺) calculated for [C₂₁H₁₃N₄O₂]⁺ 353.1039, found 353.1045. *Missing ¹³C NMR, not measured at time and no remaining sample.*

Compound 32



Pyridine·HCl (5.00 g) was added to a round bottom flask and heated to 200 °C for 30 mins. Next, compound **28** (400 mg, 0.633 mmol) was added and the reaction stirred for 1 hour before being cooled to room temperature. The reaction mixture was filtered and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL). The product was purified via column chromatography (silica, 50:50 DCM:MeCN, 20 mM NaPF₆) to afford **32** in 60% yield (222 mg, 0.0380 mmol). ¹H NMR (400 MHz, Acetonitrile-d₃) δ /ppm 8.22 (t, J = 8.6 Hz, 1H), 8.06 (td, J = 8.5, 5.7 Hz, 2H), 7.57 (dd, J = 8.8, 4.5 Hz, 2H), 7.55 – 7.46 (m, 2H), 7.28 (dd, J = 8.1, 1.2 Hz, 2H), 4.86 (t, J = 6.9 Hz, 2H), 4.60 (t, J = 7.1 Hz, 2H), 4.08 (t, J = 6.9 Hz, 2H), 3.60 (t, J = 4.6 Hz, 4H), 2.85 (t, J = 7.1 Hz, 2H), 2.58 (t, J = 4.6 Hz, 4H). *Missing ¹³C NMR, accurate mass measurement, not measured at time and no remaining sample.*

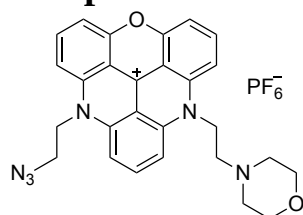
Compound 33



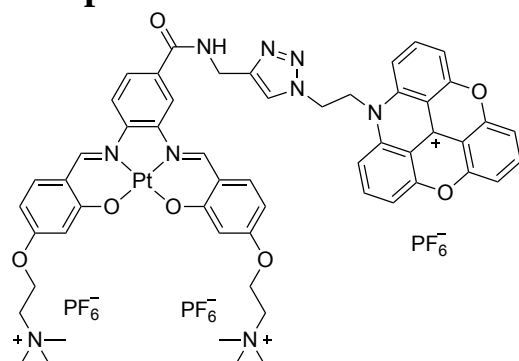
A solution of **32** (50.0 mg, 0.101 mmol) in DCM (20 mL) was cooled to 0 °C followed by the addition of triethylamine (59.8 μ L, 0.432 mmol) and tosyl anhydride (70.4 mg, 0.216 mmol). The mixture was stirred for 4 hours. Compound **33** precipitated out as the tosylate salt in

48% yield. ^1H NMR (400 MHz, DMSO- d_6) δ /ppm 8.30 (t, J = 8.6 Hz, 1H), 8.24 (t, J = 8.5 Hz, 1H), 8.12 (t, J = 8.5 Hz, 1H), 7.82 (d, J = 8.9 Hz, 1H), 7.72 – 7.61 (m, 3H), 7.53 – 7.43 (m, 4H), 7.16 – 7.10 (m, 2H), 7.04 – 7.00 (m, 2H), 6.73 (d, J = 8.0 Hz, 2H), 5.01 (s, 2H), 4.83 (s, 2H), 4.71 (s, 2H), 3.59 (t, J = 4.6 Hz, 4H), 2.88 (t, J = 6.6 Hz, 2H), 2.64 (s, 4H). *Missing ^{13}C NMR, accurate mass measurement, not measured at time and no remaining sample.*

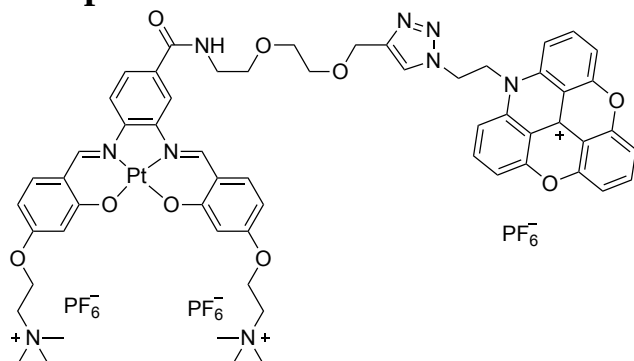
Compound 34



Compound **33** (100 mg, 0.131 mmol) and sodium azide (25.5 mg, 0.392 mmol) were combined in DMSO (0.5 mL) and stirred for 48 hours. Subsequent addition of a sat. aq. solution of NaPF_6 (5 mL) caused the compound to precipitate, after which it was filtered and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL) before being dried under reduced pressure. The product was purified via column chromatography (silica, 50:50 DCM:MeCN, 20 mM NaPF_6) to afford **33** in 89% yield (70.9 mg, 0.116 mmol). ^1H NMR (400 MHz, Acetonitrile- d_3) δ /ppm 8.27 (t, J = 8.7 Hz, 1H), 8.15 – 8.05 (m, 2H), 7.65 – 7.56 (m, 2H), 7.53 (dd, J = 8.7, 2.9 Hz, 2H), 7.31 (ddd, J = 8.2, 2.5, 0.6 Hz, 2H), 4.71 (t, J = 6.1 Hz, 2H), 4.62 (t, J = 6.9 Hz, 2H), 4.00 (t, J = 6.1 Hz, 2H), 3.65 – 3.58 (m, 4H), 2.89 (t, J = 7.0 Hz, 2H), 2.63 – 2.57 (m, 4H). ^{13}C NMR (101 MHz, DMSO- d_6) δ /ppm 152.13, 151.98, 141.36, 140.66, 140.41, 139.84, 139.60, 138.84, 138.62, 111.43, 110.62, 109.99, 108.67, 108.54, 107.33, 107.14, 106.41, 66.62, 57.96, 54.04, 49.90. HPLC-MS(ESI) calculated for $[\text{M}]^+$ 465.2, found 465.1. TOF MS (ES^+) calculated for $[\text{C}_{27}\text{H}_{25}\text{N}_6\text{O}_2]^+$ 465.2049, found 365.2041.

Compound 35

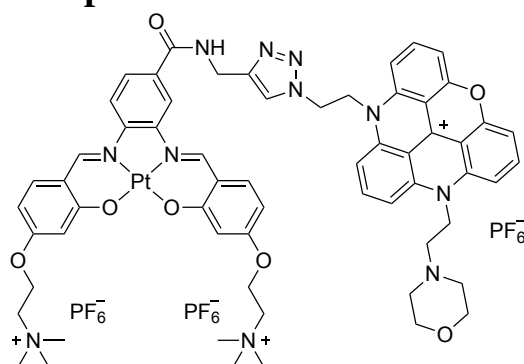
Compound **15** (34.8 mg, 0.0321 mmol), compound **31** (20 mg, 0.0401 mmol), $[\text{Cu}(\text{MeCN})_4] \cdot \text{PF}_6$ (14.9 mg, 0.0401 mmol) and DIPEA (6.99 μL , 0.0401 mmol) were combined in acetonitrile and stirred overnight. The desired compound was precipitated out with DCM and purified via RP-HPLC. ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 9.43 (s, 1H), 9.27 (s, 1H), 9.03 (s, 1H), 8.67 (s, 1H), 8.43 (d, $J = 8.9$ Hz, 1H), 8.29 (t, $J = 8.5$ Hz, 2H), 8.06 (t, $J = 8.4$ Hz, 1H), 7.94 (s, 1H), 7.86 – 7.70 (m, 6H), 7.55 (dd, $J = 10.8, 8.3$ Hz, 5H), 6.73 (d, $J = 2.4$ Hz, 2H), 6.57 (m, 2H), 5.37 (s, 2H), 5.03 (s, 2H), 4.59 (s, 6H), 4.43 – 4.33 (m, 2H), 3.85 (t, $J = 4.7$ Hz, 6H), 3.21 (s, 18H). HPLC-MS(ESI) calculated for $[\text{M}]^{3+}$ 382.5, found 382.7. *Missing ^{13}C NMR, insufficient compound.*

Compound 36

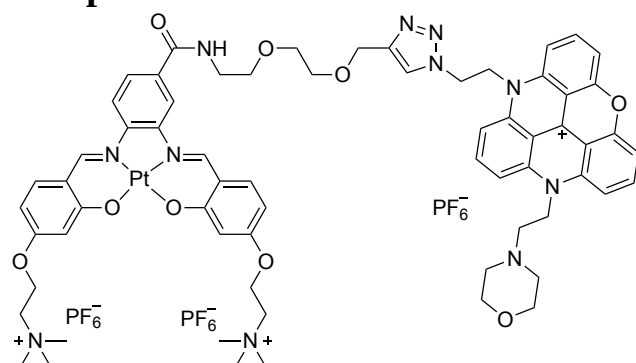
Compound **25** (37.7 mg, 0.0321 mmol), compound **31** (20 mg, 0.0401 mmol), $[\text{Cu}(\text{MeCN})_4] \cdot \text{PF}_6$ (14.9 mg, 0.0401 mmol) and DIPEA (6.99 μL , 0.0401 mmol) were combined in acetonitrile and stirred overnight. The desired compound was precipitated out with DCM and purified via RP-HPLC. ^1H NMR (400 MHz, DMSO-d_6) δ/ppm 8.92 (s, 1H), 8.80 (s, 1H), 8.46 (t, $J = 5.6$ Hz, 1H), 8.34 (s, 1H), 8.19 (s, 1H), 8.13 (d, $J = 8.9$ Hz, 1H), 7.90 (t, $J = 8.5$ Hz, 2H), 7.80 (t, $J = 8.4$ Hz, 1H), 7.65 (dd, $J = 16.6, 9.0$ Hz, 2H), 7.55 (d, $J = 9.6$ Hz,

1H), 7.26 (dd, $J = 11.3, 8.3$ Hz, 4H), 7.13 (d, $J = 9.0$ Hz, 2H), 6.34 – 6.44 (m, 4H), 4.83 (s, 4H), 4.56 (d, $J = 14.5$ Hz, 4H), 4.46 (s, 2H), 3.87 (s, 4H), 3.73 – 3.64 (m, 4H), 3.62 – 3.53 (m, 4H), 3.24 (s, 18H). ^{13}C NMR (101 MHz, DMSO- d_6) δ /ppm 166.90, 166.47, 165.49, 164.09, 163.77, 152.56, 151.92, 149.97, 149.48, 145.89, 145.26, 143.59, 140.83, 139.81, 137.30, 137.14, 125.93, 125.66, 117.00, 115.64, 111.58, 109.65, 109.36, 108.72, 108.55, 108.07, 105.75, 103.44, 70.11, 69.57, 69.26, 64.39, 63.66, 62.25, 53.70, 49.06, 47.99, 47.11, 31.16. HPLC-MS(ESI) calculated for $[\text{M}]^{3+}$ 411.8, found 412.0.

Compound 37



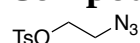
Compound **15** (28.4 mg, 0.0262 mmol), compound **34** (20 mg, 0.0327 mmol), $[\text{Cu}(\text{MeCN})_4] \cdot \text{PF}_6$ (12.2 mg, 0.0327 mmol) and DIPEA (5.71 μL , 0.0327 mmol) were combined in acetonitrile and stirred overnight. The desired compound was precipitated out with DCM and purified via RP-HPLC. ^1H NMR (400 MHz, DMSO- d_6) δ /ppm 9.44 (s, 1H), 9.29 (s, 1H), 9.12 (s, 1H), 8.69 (s, 1H), 8.43 (d, $J = 8.9$ Hz, 1H), 8.26 (t, $J = 8.4$ Hz, 1H), 8.05 (q, $J = 8.9, 7.6$ Hz, 2H), 7.82 (dd, $J = 14.9, 9.0$ Hz, 3H), 7.67 (d, $J = 9.1$ Hz, 1H), 7.48 (d, $J = 9.2$ Hz, 1H), 7.38 (dd, $J = 21.2, 8.1$ Hz, 2H), 6.74 (d, $J = 2.5$ Hz, 2H), 6.57 (ddd, $J = 13.8, 8.9, 2.5$ Hz, 2H), 5.17 (s, 2H), 5.00 (s, 2H), 4.83 (s, 2H), 4.59 (s, 4H), 4.44 (d, $J = 5.6$ Hz, 2H), 3.86 (d, $J = 4.9$ Hz, 4H), 3.22 (s, 18H). TOF MS (ES^+) calculated for $[\text{C}_{61}\text{H}_{66}\text{N}_{11}\text{O}_7\text{Pt}]^{3+}$ 420.1604, found 420.1620. HPLC-MS(ESI) calculated for $[\text{M}]^{3+}$ 420.2, found 420.2. *Missing ^{13}C NMR, insufficient compound.*

Compound 38

Compound **25** (30.7 mg, 0.0262 mmol), compound **34** (20 mg, 0.0327 mmol), $[\text{Cu}(\text{MeCN})_4] \cdot \text{PF}_6$ (12.2 mg, 0.0327 mmol) and DIPEA (5.71 μL , 0.0327 mmol) were combined in acetonitrile and stirred overnight. The desired compound was precipitated out with DCM and purified via RP-HPLC. ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 8.84 (d, J = 17.8 Hz, 2H), 8.44 (t, J = 5.6 Hz, 1H), 8.38 (s, 1H), 8.21 (s, 1H), 8.06 (d, J = 8.9 Hz, 1H), 7.88 – 7.76 (m, 1H), 7.80 – 7.69 (m, 2H), 7.62 (d, J = 9.2 Hz, 2H), 7.52 (d, J = 9.6 Hz, 1H), 7.31 (d, J = 8.9 Hz, 1H), 7.15 – 7.04 (m, 2H), 6.94 (d, J = 8.9 Hz, 1H), 6.83 (d, J = 8.4 Hz, 1H), 6.57 – 6.36 (m, 4H), 4.80 (s, 2H), 4.67 (s, 2H), 4.53 (d, J = 15.0 Hz, 8H), 4.44 (s, 2H), 4.08 (q, J = 5.2 Hz, 1H), 3.86 (s, 4H), 3.68 (t, J = 4.7 Hz, 4H), 3.64 – 3.51 (m, 8H), 3.23 (s, 18H), 3.27 – 3.13 (m, 8H), 2.75 (s, 2H), 2.56 (s, 4H). ^{13}C NMR (101 MHz, DMSO-d_6) δ /ppm 164.98, 163.55, 151.57, 149.34, 148.96, 140.19, 139.74, 139.06, 138.13, 132.94, 125.08, 116.92, 110.42, 108.06, 107.27, 102.99, 69.61, 69.08, 68.72, 66.22, 63.90, 63.25, 61.76, 53.45, 53.21, 48.62, 39.98, 30.73. TOF MS (ES^+) calculated for $[\text{C}_{65}\text{H}_{74}\text{N}_{11}\text{O}_9\text{Pt}]^{3+}$ 449.5111, found 449.5133. HPLC-MS(ESI) calculated for $[\text{M}]^{3+}$ 449.2, found 449.3.

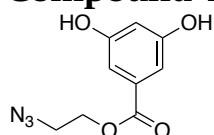
7.2.3 Chapter 4

Compounds **43**, **44** and **45** were provided by Dr James Lewis.

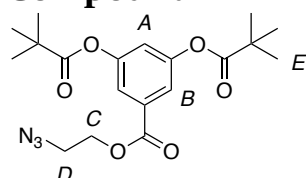
Compound 39*

Compound synthesised according to established protocols.^[263] 2-Azidoethanol (1.4 g, 16.1 mmol), tosyl chloride (6.13 g, 32.2 mmol) and triethylamine (4.46 mL, 32.2 mmol) were

combined in DCM (25 mL) and stirred overnight at room temperature. The solvent was removed and the compound purified via column chromatography (silica, 100% DCM). ^1H NMR (400 MHz, Chloroform- d) δ /ppm 7.86 – 7.76 (m, 2H), 7.39 – 7.33 (m, 2H), 4.16 (t, J = 5.8 Hz, 2H), 3.49 (t, J = 5.8 Hz, 2H), 2.46 (s, 3H).

Compound 40*

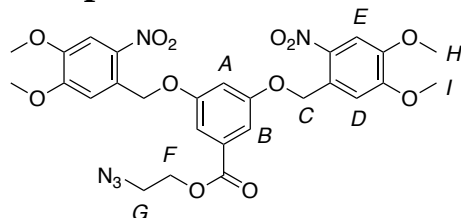
Compound synthesised according to established protocols.^[263] Compound **39** (500 mg, 2.07 mmol), potassium carbonate (401 mg, 2.90 mmol) and 3,5 dihydroxybenzoic acid (287 mg, 1.86 mmol) were combined in DMF (20 mL) and stirred overnight. The reaction mixture was poured in 50 mL water and was extracted with DCM (3 x 50 mL). The solvent was removed under reduced pressure and purified via column chromatography (silica, 9:1 DCM:MeOH). ^1H NMR (400 MHz, Chloroform- d) δ /ppm 8.45 (s, 2H), 7.06 (d, J = 2.3 Hz, 2H), 6.64 (t, J = 2.3 Hz, 1H), 4.43 – 4.36 (m, 2H), 3.57 – 3.51 (m, 2H).

Compound 41

A solution of EDC·HCl (387 mg, 2.02 mmol) and pivalic acid (228 mg, 2.24 mmol) in DCM (30 mL) was cooled to 0 °C for 30 mins, followed by the addition of compound **40** (200 mg, 0.896 mmol) and DMAP (246 mg, 2.02 mmol). The solution was allowed to warm to room temperature and stirred for 1.5 hours. Next, a solution of v/v 5% citric acid (aq.) (20 mL) was added and the organic layer extracted with DCM (3 x 20 mL). The organic layer was concentrated under reduced pressure, dried with MgSO_4 and purified via column chromatography (Silica, hexane:ethyl acetate, 50:50) to afford compound **41** as a pale yellow oil in 51% yield (157 mg, 0.457 mmol). ^1H NMR (400 MHz, Chloroform- d) δ /ppm 7.62 (d, J = 2.2 Hz, 2H, B), 7.10 (t, J = 2.2 Hz, 1H, A), 4.53 – 4.46 (m, 2H, C), 3.62 – 3.57 (m, 2H, D), 1.35 (s, 18H, E). ^{13}C NMR (101 MHz, Chloroform- d) δ /ppm 176.50, 164.71, 151.51, 131.44,

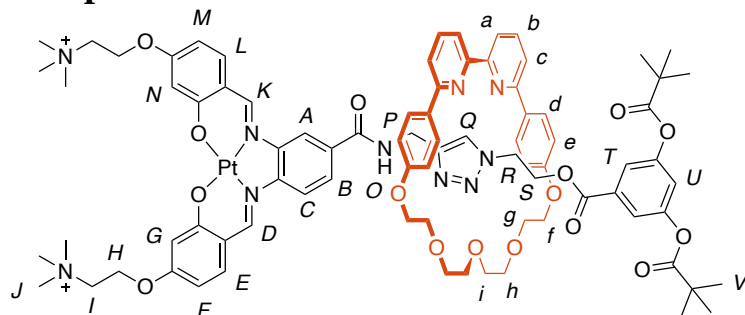
120.64, 120.26, 64.12, 49.84, 39.17, 26.52. TOF MS (ES^+) calculated for $[\text{C}_{19}\text{H}_{25}\text{N}_3\text{O}_6\text{Na}]^+$ 414.1641, found 414.1635.

Compound 42



Compound **40** (200 mg, 0.896 mmol), 4,5-dimethoxy-2-nitrobenzyl bromide (520 mg, 1.88 mmol) and K_2CO_3 (310 mg, 2.24 mmol) were dissolved in DMF (10 mL). The reaction mixture was stirred at room temperature in the dark for two days. The precipitate formed was collected, washed with water and dried under vacuum to afford compound **42** as a white solid in 85% yield (467 mg, 0.762 mmol), which was used without further purification. ^1H NMR (400 MHz, Chloroform- d) δ /ppm 7.80 (s, 2H, E), 7.39 (d, $J = 2.4$ Hz, 2H, B), 7.32 (s, 2H, D), 6.92 (t, $J = 2.3$ Hz, 1H, A), 5.57 – 5.52 (m, 4H, C), 4.57 – 4.49 (m, 2H, F), 4.01 (d, $J = 2.0$ Hz, 12H, I, H), 3.65 – 3.58 (m, 2H, G). ^{13}C NMR (101 MHz, Chloroform- d) δ /ppm 165.56, 159.33, 153.95, 148.03, 139.18, 131.89, 128.39, 109.46, 109.32, 108.11, 107.42, 67.43, 64.31, 56.54, 56.43, 49.90. TOF MS (ES^+) calculated for $[\text{C}_{19}\text{H}_{25}\text{N}_3\text{O}_6\text{Na}]^+$ 414.1641, found 414.1635.

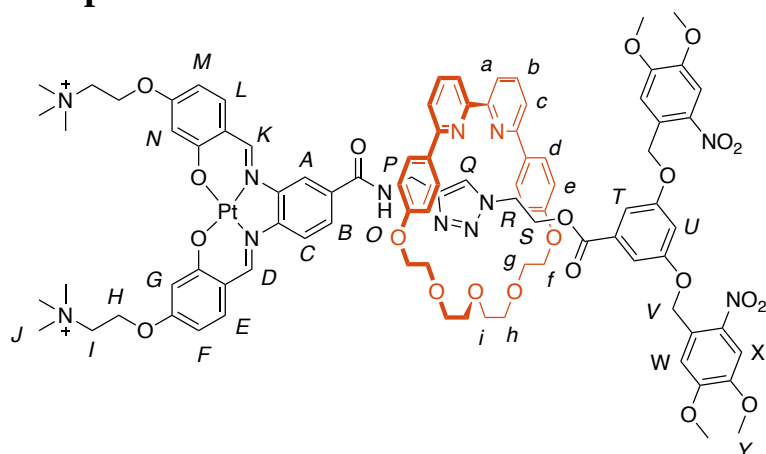
Compound 46^{enz}



Macrocycle **45** (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by $[\text{Cu}(\text{CH}_3\text{CN})_4](\text{PF}_6)$ (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen **15** (50 mg, 0.05 mmol) and stopper **41** (18.0 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer

was separated, dried using MgSO_4 and the solvent removed under reduced pressure. The resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF_6) to afford **46**^{enz} in 10% yield as an orange solid (9.10 mg, 0.005 mmol). ^1H NMR (400 MHz, DMSO-d_6) δ /ppm 9.39 (s, 1H, D), 8.76 (s, 1H, K), 8.60 (s, 1H, A), 8.12 – 8.01 (m, 6H, a/b/c), 7.78 (s, 1H, Q), 7.63 – 7.55 (m, 3H, C, E, L), 7.42 (d, $J = 2.2$ Hz, 2H, T), 7.37 (d, $J = 8.7$ Hz, 1H, B), 7.32 (t, $J = 2.2$ Hz, 1H, U), 6.94 (d, $J = 8.2$ Hz, 4H, d), 6.67 (d, $J = 2.4$ Hz, 1H, G), 6.62 (d, $J = 2.4$ Hz, 1H, N), 6.46 (dd, $J = 8.9, 2.4$ Hz, 1H, F), 6.36 (s, 1H, O), 6.25 – 6.14 (m, 5H, M, e), 4.66 (t, $J = 5.2$ Hz, 2H, P), 4.59 – 4.46 (m, 6H, H, R), 3.96 – 3.87 (m, 2H, macrocycle), 3.83 (t, $J = 5.2$ Hz, 4H, I), 3.79 (t, $J = 3.4$ Hz, 2H, S), 3.76 – 3.61 (m, 10H, macrocycle), 3.54 – 3.44 (m, 4H, macrocycle), 3.20 – 3.15 (m, 18H, J), 1.29 (s, 18H, V). ^{13}C NMR (101 MHz, DMSO-d_6) δ /ppm 176.49, 166.54, 166.29, 164.46, 164.31, 163.72, 163.40, 160.41, 158.81, 156.76, 151.75, 145.37, 144.45, 143.05, 139.10, 133.42, 131.72, 129.03, 123.73, 121.65, 121.42, 120.44, 114.03, 108.44, 107.77, 103.94, 70.83, 70.46, 69.13, 67.26, 64.41, 62.11, 53.61, 29.47, 27.12.

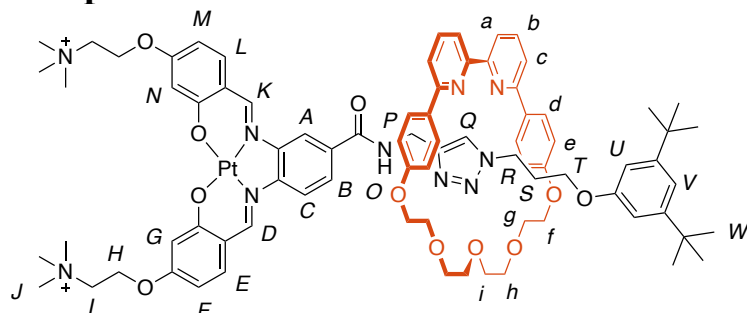
Compound **46**^{photo}



Macrocycle **45** (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by $[\text{Cu}(\text{CH}_3\text{CN})_4](\text{PF}_6)$ (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen **15** (50 mg, 0.05 mmol) and stopper **3** (28.3 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 $\text{NH}_4\text{OH}/\text{H}_2\text{O}$ with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer was separated, dried using MgSO_4 and the solvent removed under reduced pressure. The

resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF₆) to afford rotaxane **46**^{photo} in 9% yield as an orange solid (9.11 mg, 0.004 mmol). ¹H NMR (500 MHz, DMSO-d₆) δ/ppm 9.40 (s, 1H, D), 8.78 (s, 1H, K), 8.62 (s, 1H, A), 8.16 – 8.01 (m, 6H, a, b, c), 7.69 (s, 1H, Q), 7.67 – 7.61 (m, 2H, E, L), 7.59 (d, J = 9.0 Hz, 1H, C), 7.39 (d, J = 9 Hz, 1H, B), 6.98 (d, J = 8.1 Hz, 4H, d), 6.94 (t, J = 1.6 Hz, 1H, V), 6.68 – 6.61 (m, 4H, G, N, U), 6.47 (dd, J = 9, 2.4 Hz, 1H, F), 6.40 (s, 1H, O), 6.22 (m, 5H, M, e), 4.56 – 4.47 (m, 4H, H), 4.38 (t, J = 7.1 Hz, 2H, R), 3.95 – 3.89 (m, 2H, macrocycle), 3.89 – 3.76 (m, 8H, I, T, macrocycle), 3.75 – 3.68 (m, 2H, macrocycle), 3.61 (s, 4H, macrocycle), 3.50 (s, 4H, macrocycle), 3.30 (s, 2H, macrocycle), 3.18 (m, 18H, J), 2.08 (q, J = 6.5 Hz, 2H, P), 1.22 (s, 18H, W). TOF MS (ES⁺) calculated for [C₉₁H₉₈N₁₂O₂₂Pt]²⁺ 953.3296, found 953.3330. HPLC-MS(ESI) calculated for [M+H]³⁺ 635.6, found 636.1. *Missing ¹³C NMR, insufficient compound.*

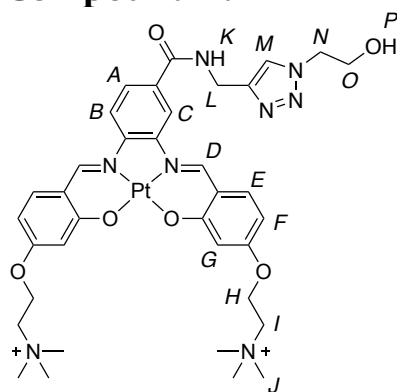
Compound **46**^{ctrl}



Macrocycle **45** (23.0 mg, 0.05 mmol) was dissolved in DMSO (2 mL), followed by [Cu(CH₃CN)₄](PF₆) (16.5 mg, 0.04 mmol). To this reaction mixture was added salphen **1** (50 mg, 0.05 mmol) and stopper **42** (13.3 mg, 0.05 mmol) and the reaction stirred for 12 hours at room temperature. Subsequently, DCM (50 mL) and a solution of v/v 1:9 NH₄OH/H₂O with 0.1 M EDTA (50 mL) was added and stirred for 1 hour. The organic layer was separated, dried using MgSO₄ and the solvent removed under reduced pressure. The resulting solid was purified via preparative thin layer chromatography (Silica, 50% acetonitrile:DCM with 50 mM NaPF₆) to afford **46**^{ctrl} in 12% yield as an orange solid (12.2 mg, 0.005 mmol). ¹H NMR (500 MHz, DMSO-d₆) δ/ppm 9.37 (s, 1H, D), 8.73 (s, 1H, K), 8.57 (s, 1H, A), 8.03 (m, 6H, a,b, c), 7.78 (s, 1H, Q), 7.70 (s, 2H, X), 7.56 (s, 3H, C, E, L), 7.33 (d, J = 9 Hz, 2H, B),

7.30 (s, 2H, W) 7.10 (d, $J = 2.2$ Hz, 2H, T), 6.95 (s, 1H, U), 6.90 (d, $J = 8.1$ Hz, 4H, e), 6.66 (s, 1H, G), 6.60 (s, 1H, N), 6.46 (d, $J = 8.8$ Hz, 1H, F), 6.21 (d, $J = 8.6$ Hz, 1H, M), 6.14 (d, $J = 8.1$ Hz, 4H, e), 5.39 (s, 4H, V), 4.66 (s, 2H, R), 4.52 (d, $J = 22.8$ Hz, 6H, P, H), 3.95 – 3.75 (m, 20H, macrocycle, Y), 3.70 (d, $J = 10.0$ Hz, 2H, macrocycle), 3.58 (m, 4H, macrocycle), 3.50 (s, 4H, macrocycle), 3.17 (m, 18H, J). ^{13}C NMR (101 MHz, DMSO- d_6) δ /ppm 166.55, 166.30, 164.42, 163.73, 163.44, 160.36, 158.86, 158.39, 156.70, 152.54, 152.10, 150.50, 145.38, 144.33, 143.08, 139.19, 136.86, 135.65, 133.37, 131.07, 129.07, 125.90, 123.32, 121.48, 120.51, 177.79, 117.52, 117.03, 166.45, 114.85, 114.07, 109.11, 108.15, 107.74, 103.87, 79.42, 79.09, 70.81, 70.45, 70.25, 69.13, 67.28, 64.45, 62.12, 53.60, 46.80, 35.03, 34.60, 31.65, 30.19, 29.47. HPLC-MS(ESI) calculated for $[\text{M}+\text{H}]^{3+}$ 527.7, found 527.7.

Compound 47

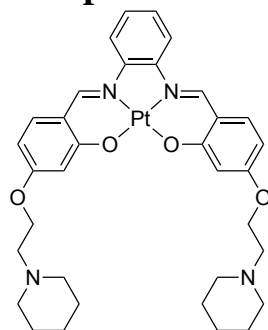


Salphen **15** (50 mg, 0.05 mmol) and azidoethanol (6.02 mg, 0.07 mmol) were dissolved in DMSO (2 mL). This was followed by the addition of $[\text{Cu}(\text{CH}_3\text{CN})_4](\text{PF}_6)$ (3.42 mg, 0.009 mmol). The reaction was stirred at room temperature for 12 hours before precipitation with DCM (20 mL). The solid was filtered and washed a further 3 times with DCM (10 mL) and purified by RP-HPLC to afford **47** in 60% yield as an orange solid (32.4 mg, 0.277 mmol). ^1H NMR (400 MHz, DMSO- d_6) δ /ppm 9.41 (m, 2H, D), 9.19 (t, $J = 5.7$ Hz, 1H, K), 8.83 (s, 1H, C), 8.44 (d, $J = 9.0$ Hz, 1H, B), 7.98 (s, 1H, M), 7.90 (d, $J = 9$ Hz, 1H, A), 7.88 – 7.78 (m, 2H, F), 6.73 – 6.67 (m, 2H, G), 6.58 – 6.52 (m, 2H, E), 5.04 (t, $J = 5.4$ Hz, 1H, P), 4.65 – 4.50 (m, 6H, H, L), 4.39 (t, $J = 5.4$ Hz, 2H, N), 3.83 (s, 4H, I), 3.77 (q, $J = 5.4$ Hz, 2H, O), 3.19 (s, 18H, J). ^{13}C NMR (101 MHz, DMSO- d_6) δ /ppm 166.64, 166.47, 164.88, 163.74, 163.57, 150.70, 150.31, 146.39, 144.49, 144.14, 137.16, 132.60, 125.87, 123.54, 118.13, 117.19, 116.01, 115.72, 108.34, 108.07,

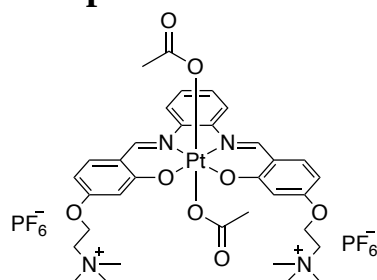
7.2.4 Chapter 5

The chemical structure shows a central Pt(II) complex. The platinum atom is coordinated by two nitrogen atoms of a 2,2',6,6'-tetraakis(4-ethoxyphenyl)-2,6-pyridinediyl ligand and two oxygen atoms of another identical ligand. The ligands are connected via their phenyl rings to ethoxy chains, which are further connected to quaternary ammonium cations (N⁺). Two PF₆⁻ counterions are shown near the ammonium groups.

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Compound 49*

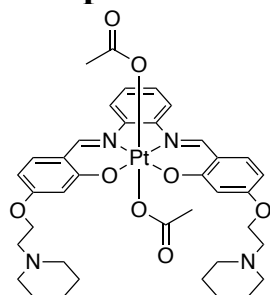
Compound synthesised according to established protocols.^[172] Diaminobenzene (30 mg, 0.277 mmol) and compound **3** (138 mg, 0.554 mmol) were combined in MeOH (5 mL) and stirred under reflux for 4 hours. The solvent was removed, and the residue redissolved in DMSO (1 mL). Sodium acetate (47.8 mg, 0.583 mmol), followed by K₂PtCl₄ (126 mg, 0.305 mmol) was added and the temperature increased to 80 °C and stirred overnight. The resulting precipitate was filtered. This product was dissolved in water, followed by the addition of a sat. aq. solution of NaPF₆. This precipitate was filtered and washed with water (3 x 10 mL) and diethyl ether (3 x 10 mL) before being dried under reduced pressure to give compound **49**. ¹H NMR (400 MHz, DMSO-d₆) δ/ppm 9.34 (s, 2H), 8.37 (dt, J = 7.2, 3.6 Hz, 2H), 7.77 (d, J = 9.0 Hz, 2H), 7.40 (dt, J = 7.1, 3.5 Hz, 2H), 6.64 (s, 2H), 6.52 (dd, J = 8.9, 2.5 Hz, 2H), 4.30 (s, 4H), 3.20 - 3.50 (m, 12H) 1.64 (s, 8H), 1.47 (s, 4H).

Compound 50

Compound **48** (20 mg, 0.0200 mmol) was dissolved in acetonitrile. Diacetoxyiodobenzene (38.5 mg, 0.120 mmol) was added and the reaction stirred for 16 hours. The volume of acetonitrile was reduced to 1 mL, followed by the addition of diethyl ether. The product was recrystallised from a MeCN:Et₂O using vapour diffusion. ¹H NMR (400 MHz, Acetonitrile-d₃) δ/ppm 8.62 – 8.40 (m, 2H), 8.10 – 8.02 (m, 2H), 7.66 (d, J = 9.0 Hz, 2H), 7.42 (dd, J = 6.4,

3.2 Hz, 2H), 6.74 (d, $J = 2.4$ Hz, 2H), 6.57 (dd, $J = 8.9, 2.4$ Hz, 2H), 4.55 (s, 4H), 3.74 (t, $J = 4.6$ Hz, 4H), 3.17 (s, 20H). ^{195}Pt NMR (108 MHz, Acetonitrile- d_3) δ/ppm 994.98. HPLC-MS(ESI) calculated for $[\text{M}]^{2+}$ 415.14, found 415.0. *Missing ^{13}C NMR, accurate mass measurement, insufficient compound.*

Compound 50



Diacetoxyiodobenzene (18.8 mg, 0.114 mmol) was dissolved in acetonitrile (20 mL), followed by the addition of a further 20 mL of water. Compound **49** (10 mg, 0.0189 mmol) was dissolved in acetonitrile (2 mL) and added drop-wise to the reaction mixture. After 3 hours, the acetonitrile was removed under reduced pressure until only water remained. An aq. sat. solution of NaPF_6 was added, causing the product to precipitate out. This precipitate was washed with water (3 x 5 mL) and diethyl ether (3 x 20 mL) to give an intermediate product. Leaving this product in acetonitrile overnight caused conversion to the final product, which was precipitated out via addition of diethyl ether. ^1H NMR (400 MHz, Acetonitrile- d_3) δ/ppm 8.78 – 8.57 (m, 2H), 8.19 – 8.09 (m, 2H), 7.76 (d, $J = 9.0$ Hz, 2H), 7.52 (dd, $J = 6.3, 3.3$ Hz, 2H), 6.77 (d, $J = 2.4$ Hz, 2H), 6.69 (dd, $J = 8.9, 2.4$ Hz, 2H), 4.45 (t, $J = 5.0$ Hz, 4H), 3.68 – 3.46 (m, 12H), 3.03 (d, $J = 11.6$ Hz, 4H), 1.79 (s, 12H). TOF MS (ES^+) calculated for $[\text{C}_{38}\text{H}_{48}\text{N}_4\text{O}_8\text{Pt}]^+$ 883.3120, found 883.3140. HPLC-MS(ESI) calculated for $[\text{M}+2\text{H}]^{2+}$ 441.7, found 442.0. *Missing ^{13}C NMR, insufficient compound*

7.3 DNA binding experiments

7.3.1 Oligonucleotide preparation

c-Myc and HTelo oligonucleotides (sequences below) were purchased from Eurogentec (Belgium) and *CT-DNA* was purchased from Sigma Aldrich. Stock solutions were prepared

via dilution in the appropriate buffers to concentrations of 300-400 μM determined by UV-Vis titrations using an Agilent Cary UV 60 spectrometer. The sequences used are in Table S1. Prior to use, the G4 DNA stock solutions were annealed by heating at 95 $^{\circ}\text{C}$ for 5 minutes and then cooling to room temperature overnight.

Table 7.1 G4 forming sequences used in emission titrations and CD melting experiments.

DNA	Sequence (5' to 3')
HTelo	AGGGTTAGGGTTAGGGTTAGGG
c-Myc	TGAGGGTGGGTAGGGTGGGTAA
G2T1	AGGG(TTAGGG) ₇
G2T2	AGGG(TTAGGG) ₃ TTA(TTAGGG) ₄
G2T4	AGGG(TTAGGG) ₃ (TTA) ₃ (TTAGGG) ₄
G2T6	AGGG(TTAGGG) ₃ (TTA) ₅ (TTAGGG) ₄

Table 7.2 G4 forming sequences used in FRET melting experiments.

DNA	Sequence (5' FAM to TAMRA 3')
FTHTelo	GGGTAGGGTTAGGGTTAGGG
FTc-Myc	TGAGGGTGGGTAGGGTGGGTAA
FTds26	CAATCGGATCGAATTTCGATCCGATTG

7.3.2 CD melting experiments

Experiments were carried out using a Jasco J-810 spectrophotometer using a 1 cm quartz cuvette. Temperature was controlled using a Peltier module. The signal at 295 nm was monitored as temperature was increased for 25 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ at a rate of 95 $^{\circ}\text{C}/\text{min}$. CD melting experiments were carried out with HTelo G4 DNA (100 mM NaCl, 10 mM Li cacodylate, pH 7.3). Sample preparation was carried out by combining stock solutions of ligand and DNA in the appropriate buffer. The final G4 DNA concentration in the cuvette was 5 μM and the ligand concentration was 10 μM . Melting temperatures were obtained by curve fitting in GraphPad Prism 8. The data was normalised and fitted to a variable slope Hill equation, and the melting temperature was defined at the temperature at which $y = 0.5$.

7.3.3 Fluorescence titrations

Experiments were carried out using a BMG Clariostar Microplate reader with Greiner Bio-One half volume (100 μ L/well) plates. Excitation was carried out at either 375 nm or 440 nm and recorded from 500-700 nm in addition to a matrix scan at 590 nm. Titrations were carried out using *HTelo* (Na^+) G4 DNA (100 mM NaCl, 10 mM Li cacodylate, pH 7.3), *HTelo* (K^+) G4 DNA (100 mM KCl, 10 mM Li cacodylate, pH 7.3), *c-Myc* G4 DNA (100 mM KCl, 10 mM Li cacodylate, pH 7.3) and *CT-DNA* (100 mM KCl, 10 mM Li cacodylate, pH 7.3). Ligand concentration was kept constant at 2 μ M and the following DNA equivalents were tested: 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2, 3 and 5 (for *CT-DNA* 10x base pair equivalents (BPE) were used, ie 1 to 50 BPE). Sample preparation was carried out by preparing stock solutions of double concentration ligand (4 μ M) and DNA in the appropriate buffer, before 50 μ L of each was added to the appropriate well and mixed. Experiments were conducted in triplicate and binding constants were obtained by curve fitting in Graphpad Prism 8 using Equation 1.

$$y = 0.5R[1/K + L + nx - [(1/K + L + nx)^2 - 4Lnx]^{0.5}] \quad (7.1)$$

Fluorescence response (y) was fitted against DNA concentration (x); R is the machine response, K is the association constant of ligand and DNA, L is the concentration of ligand and n is the number of binding sites per DNA (2 per G4 unit and 1 per *CT-DNA* BPE). The matrix scan results were used to plot the titration curves and 500-700 nm scan results were used to confirm that expected curve shapes and shifts were observed.

7.3.4 FRET melting

For FRET melting experiments, double dyed oligonucleotides, containing 6-carboxyfluorescein (FAM) and 5-carboxytetramethylrhodamine (TAMRA) were used. The nucleic acid stock was diluted to 20 μ M in milli-Q water, after which a further dilution was performed in the desired buffer solution to 0.4 μ M. All diluted oligonucleotides were then annealed night. The ligand was diluted to a 10 μ M stock in buffer solution. Further

dilutions were made to yield stock solutions of 0.4, 0.8, 2.0, 4.0 and 8.0 μM . To each PCR tube (Agilent Technologies), 20 μL DNA (0.4 μM) and 20 μL of the appropriate stock solution were added. A control experiment was performed, in which 20 μL buffer solution was added to the PCR tube in the place of the complex solution. Measurements were performed on a PCR Stratagene Mx3005P (Agilent Technologies) with excitation at 450–495 nm and detection at 515–545 nm. Readings were taken from 25 $^{\circ}\text{C}$ to 95 $^{\circ}\text{C}$ at interval of 0.5 $^{\circ}\text{C}$ maintaining a constant temperature for 30 seconds before each reading. Each measurement was done in triplicate.

7.3.5 FID assay

Experiments were carried out using a BMG Clariostar Microplate reader with Greiner Bio-One half volume (100 μL well) plates. Excitation was carried out at 475 nm and emission was recorded at 520 nm. Titrations were carried out using c-Myc G4 DNA (100 mM KCl, 10 mM TRIS-HCl, pH 7.3). The final concentrations in the plate were 1 μM DNA, 2 μM thiazole orange (TO) and 0 – 20 μM salphen (0, 0.9375, 1.25, 1.875, 2.5, 3.75, 5, 7.5, 10, 15, 20). Sample preparation was carried out by first preparing double concentration stocks of the compound (40 and 30 μM) and a DNA/TO mixture (2 μM and 4 μM respectively). Salphen concentrations were prepared using serial dilutions at 50 μL per well, which was followed by the addition of 50 μL of the DNA/TO mixture. The mixture was gently shaken for 5 minutes before measurements were recorded.

7.4 Rotaxane stability and activation experiments

7.4.1 Buffer stability

A solution of the appropriate compound in PBS buffer was incubated at 37 $^{\circ}\text{C}$ for 24 hours, after which analysis was carried out via HPLC.

7.4.2 Human serum stability

A solution of the appropriate compound in human serum was prepared and incubated at 37 °C. At prescribed timepoints (1, 3, 6 and 24 hours) a sample was removed and diluted with 3x volume of ice-cold acetonitrile and kept on ice for 30 minutes for protein precipitation. After 30 minutes the sample was centrifuged, and the supernatant was removed and analysed via HPLC.

7.4.3 Esterase activation

A solution of rotaxane **46**^{enz} was prepared in PBS buffer pH 7.3, to which was added 50 U/μmol of porcine liver esterase (PLE). The resulting solution was incubated at 37 °C. At prescribed timepoints (1, 3, 6 and 24 hours) a sample was removed and heated for 5 minutes at 95 °C to denature the enzyme, after which 1x volume of acetonitrile was added. The sample was centrifuged, and the supernatant was removed and analysed via HPLC.

7.4.4 Photoactivation

A solution of **46**^{photo} was prepared in PBS buffer pH 7.3 and placed 5 cm from a 365 nm light source in a UV box. The solution was irradiated at different time points (0, 5, 10, 20 and 60 minutes), after which 1x volume of acetonitrile was added and the sample kept in the dark until HPLC analysis.

7.4.5 Fluorescence titrations

Titration were carried out using *HTelo* G4 DNA, *c-Myc* G4 DNA and *CT-DNA* (PBS buffer, pH 7.3 for all). Ligand concentration was kept constant at 2 μM and prepared via direct dilution of a solution of **46**^{enz} incubated in buffer for 24 hours, **46**^{enz} incubated for 24 hours with 50 U/μM of PLE and **2** also incubated with PLE for 24 hours. All other parameters were identical to previous titrations.

7.5 Reduction experiments

The corresponding Pt^{II}- and Pt^{IV}-salphens were added to cuvettes at a final concentration of 2 μ M in either phenol red free buffer with 10% FBS or pH 7.3 10 mM TRIS buffer with 100 mM KCl added. To these cuvettes were added 10 equivalents of c-Myc G4 DNA (20 μ M). The fluorescence emission was measured using $\lambda_{\text{ex}} = 375$ nm and $\lambda_{\text{em}} = 500\text{--}700$ nm. Subsequently, either L-glutathione (10 mM) or ascorbic acid (10 mM) were added and the cuvettes were incubated at 37 °C. At various timepoints the emission was measured.

7.6 Cell experiments

7.6.1 Cytotoxicity

Human Bone Osteosarcoma Epithelial Cells (U2OS, from ATCC) were grown in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% foetal bovine serum at 37 °C with 5% CO₂ in humidified air. U2OS cells were seeded (5×10^3 cells, 200 μ L, 32.2 mm²) in a 96-well plate. After 24 hours a solution of an MTS/PMS mixture in fresh media was added, according to the Promega MTS assay protocol. After 4 h, absorbance at 490 nm (MTS) and 635 nm (background) was measured. Cell viability was calculated from the dose response curve of absorbance (MTS – background).

7.6.2 Live cell imaging

Human Bone Osteosarcoma Epithelial Cells (U2OS, from ATCC) were grown in high glucose Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C with 5% CO₂ in humidified air. Cells were seeded on chambered coverglass (1.5×10^4 cells, 250 μ L, 0.8 cm²) for 24 h, before washing with PBS and adding fresh media containing the compounds under study (2 or 10 μ M, 250 μ L) for a further 24h. The cells were co-stained with DAPI (50 μ M, 1 hr) and LysoTracker green (200 nM, 1 hr). For all live cell imaging, cells were mounted in the microscope stage, heated by a thermostat (Lauda GmbH, E200) to 37 °C, and kept under an atmosphere of 5% CO₂ in air. Pt-Salphen emission (525-700

nm) was collected following 458 nm excitation. DAPI emission (400–450 nm) was collected following multi-photon excitation at 760 nm with 665 and 680 nm cut off filters. Lyso-Tracker Green emission (492-525 nm) was collected following 488 nm excitation, and the same laser was used to collect the Pt-Salphen Emission (570-700 nm) simultaneously. A 100x (oil, NA = 1.4) objective was used to collect images at 512 x 512 pixel resolution.

7.6.3 Fixed cell imaging

Cells were seeded on chambered coverglass (1.5×10^4 cells, 250 μ L, 0.8 cm²) for 24 h, before washing with PBS and adding fresh media containing the compounds under study (2 μ M, 250 μ L) for a further 24h. Cells were washed (x3) in ice cold PBS before incubation in ice cold paraformaldehyde (PFA, 4% in PBS) solution for 10 min at 21 °C, and a further wash (x3) with ice cold PBS. The cells were then either kept in the dark or irradiated for 20 minutes by placing the coverglass 5 cm from a 365nm light source. The cells were then stained with DAPI (300 nM, 250 μ L) followed by a further PBS wash (3x) and the addition of mounting medium. PtII-salphen emission (525 - 700 nm) was collected following 458 nm excitation. DAPI emission (400 - 450 nm) was collected following multi-photon excitation at 760 nm with 665 and 680 nm cut off filters. A 100x (oil, NA = 1.4) objective was used to collect images at 512 x 512 pixel resolution.

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Appendices

Appendix A

Appendix

A.1 NMR spectra

A.1.1 Chapter 2

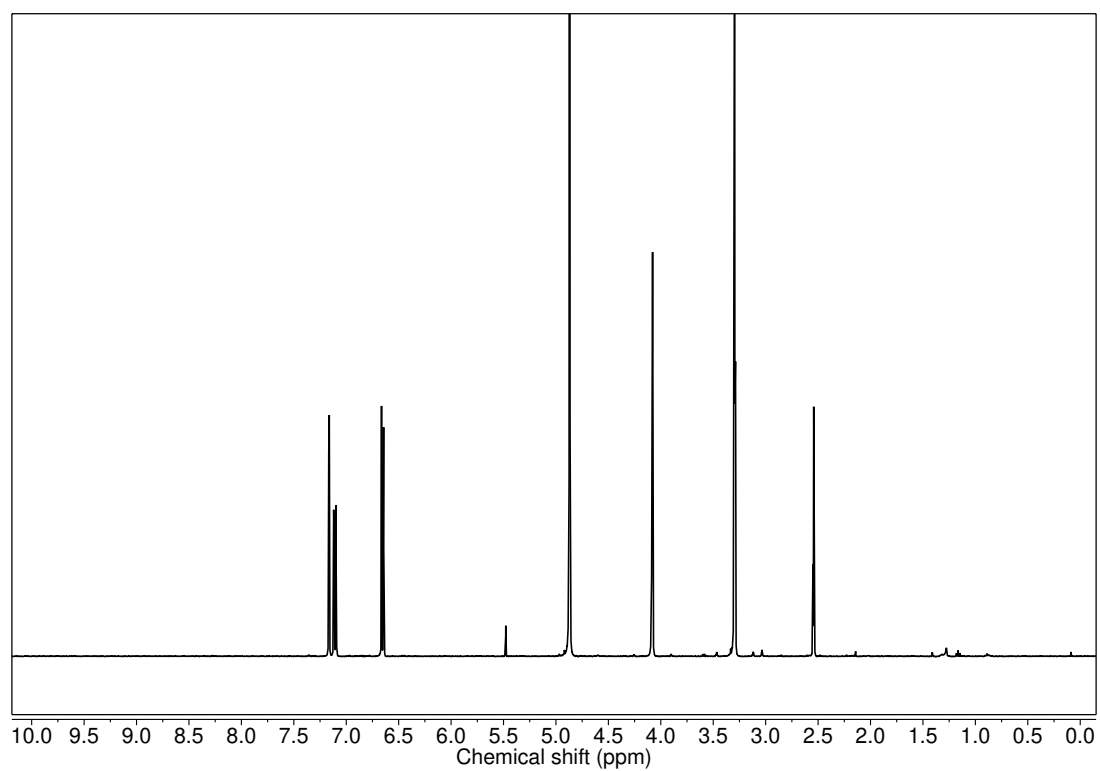


Figure A.1 ^1H NMR spectrum of compound 1 (MeOD).

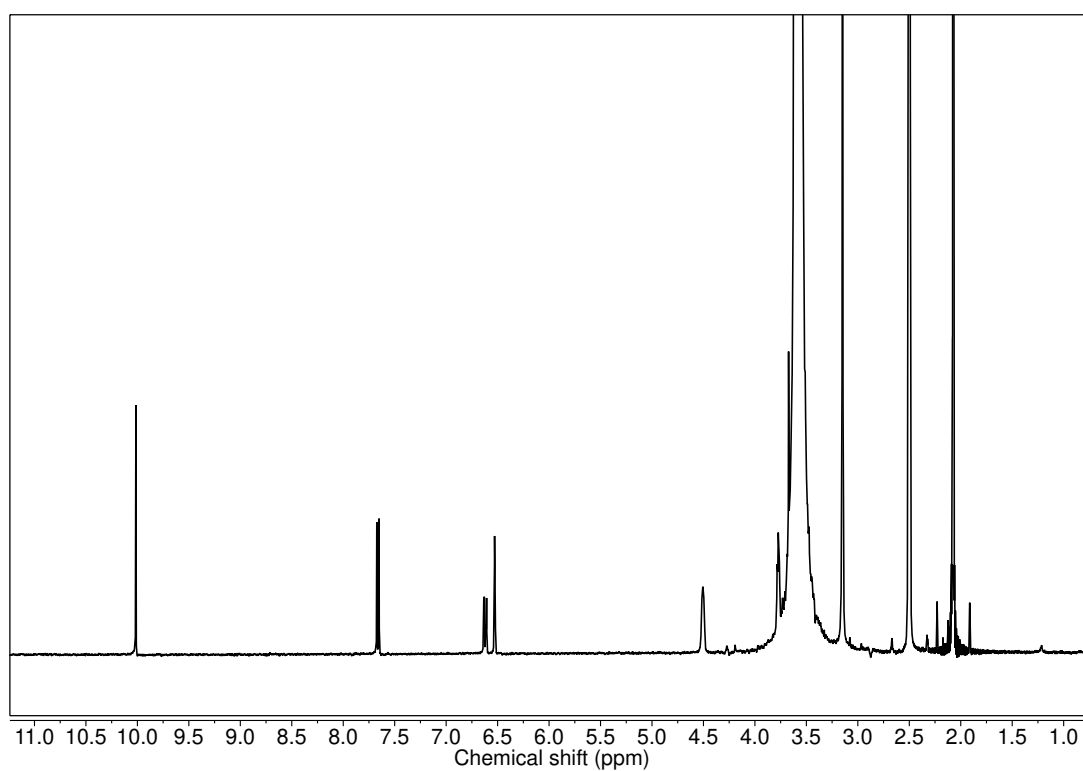


Figure A.2 ^1H NMR spectrum of compound 2 (MeOD).

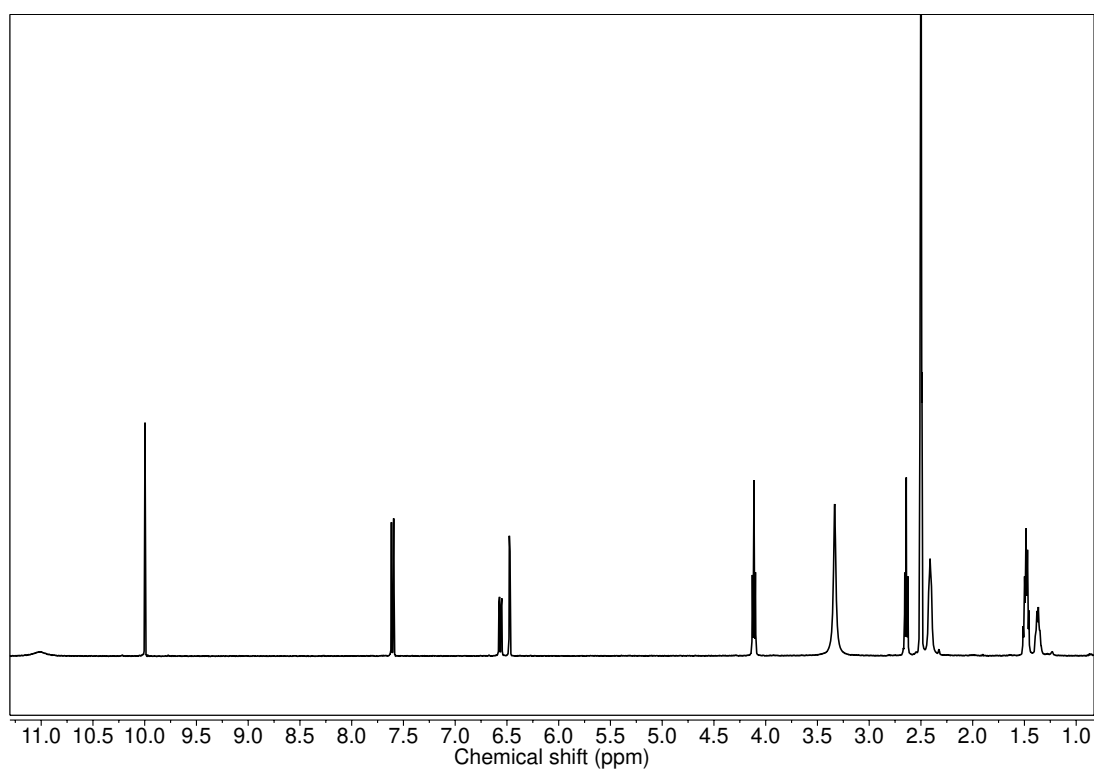


Figure A.3 ^1H NMR spectrum of compound 3 (MeOD).

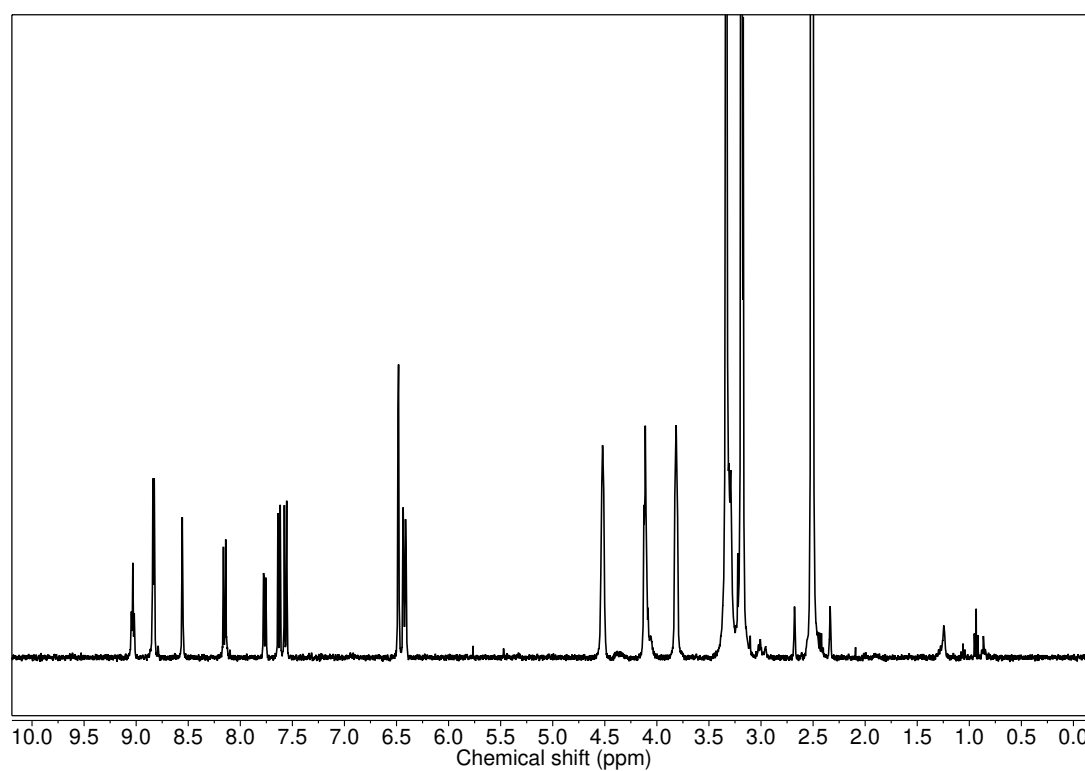


Figure A.4 ^1H NMR spectrum of compound 4 (DMSO-d_6).

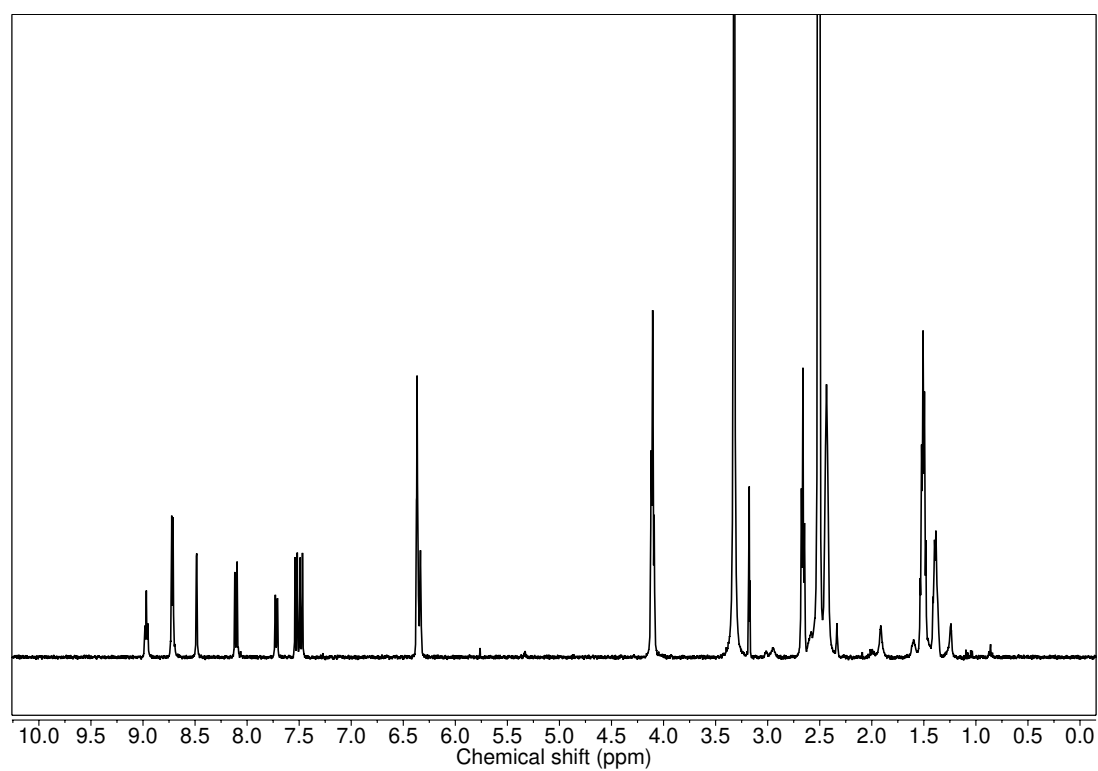


Figure A.5 ^1H NMR spectrum of compound 5 (DMSO-d_6).

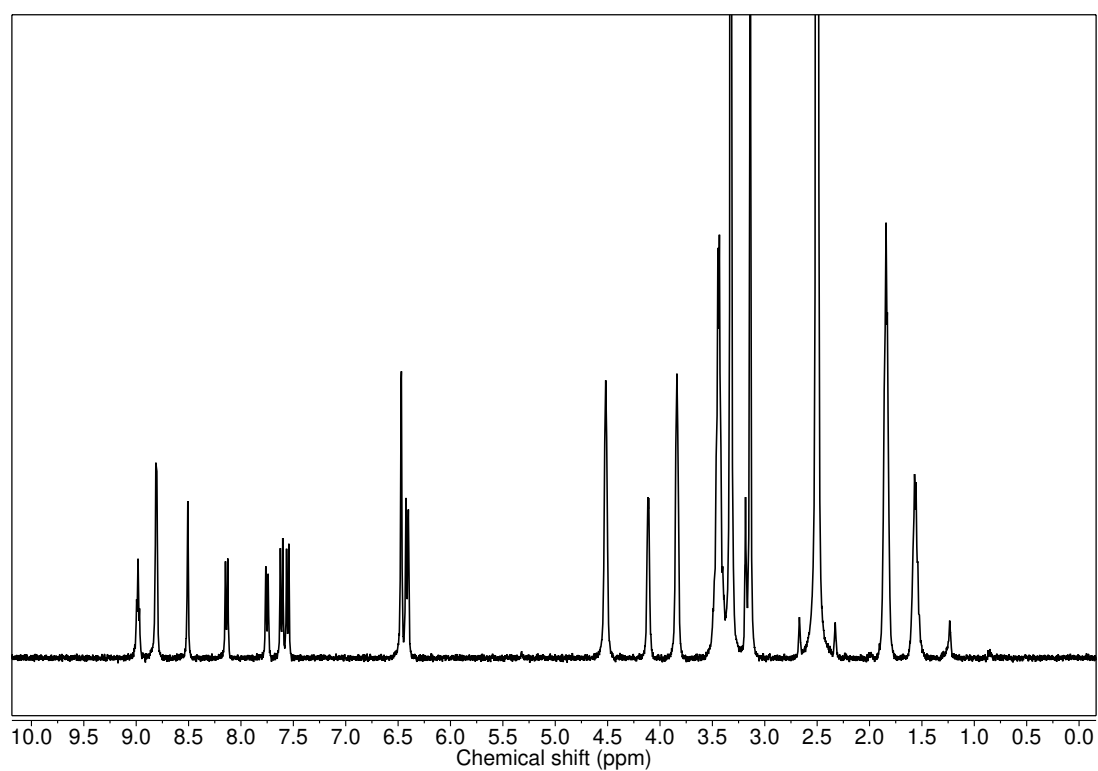


Figure A.6 ^1H NMR spectrum of compound **6** (DMSO-d_6).

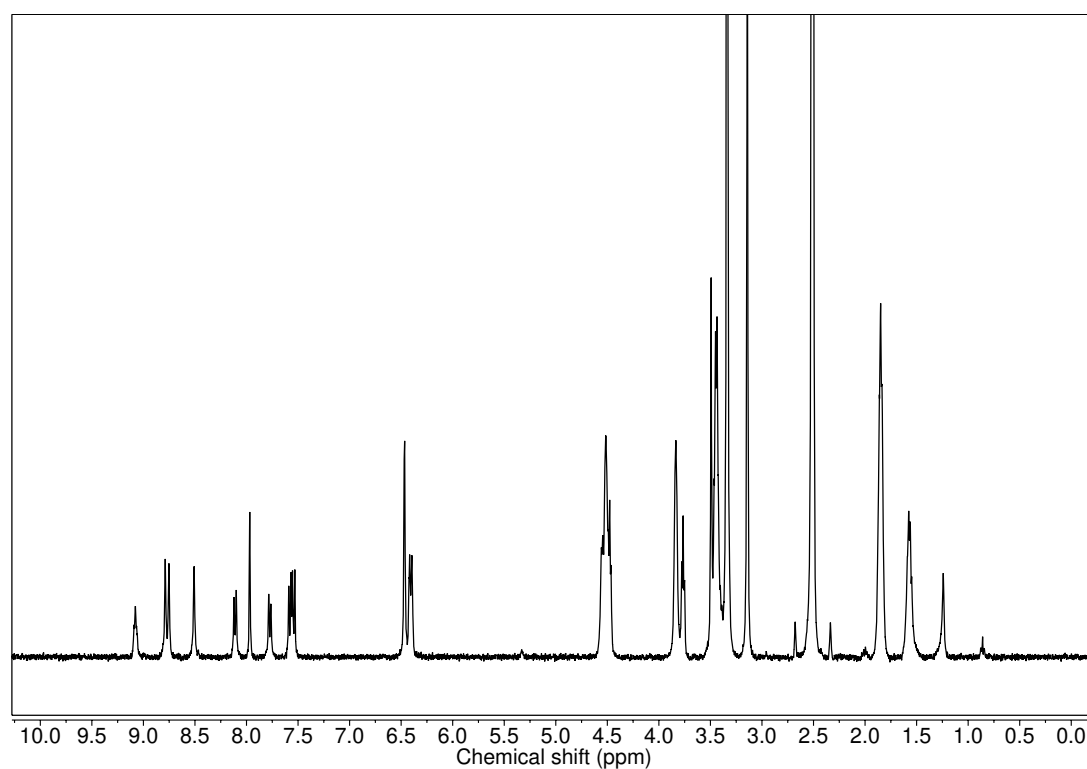


Figure A.7 ^1H NMR spectrum of compound **7** (DMSO-d_6).

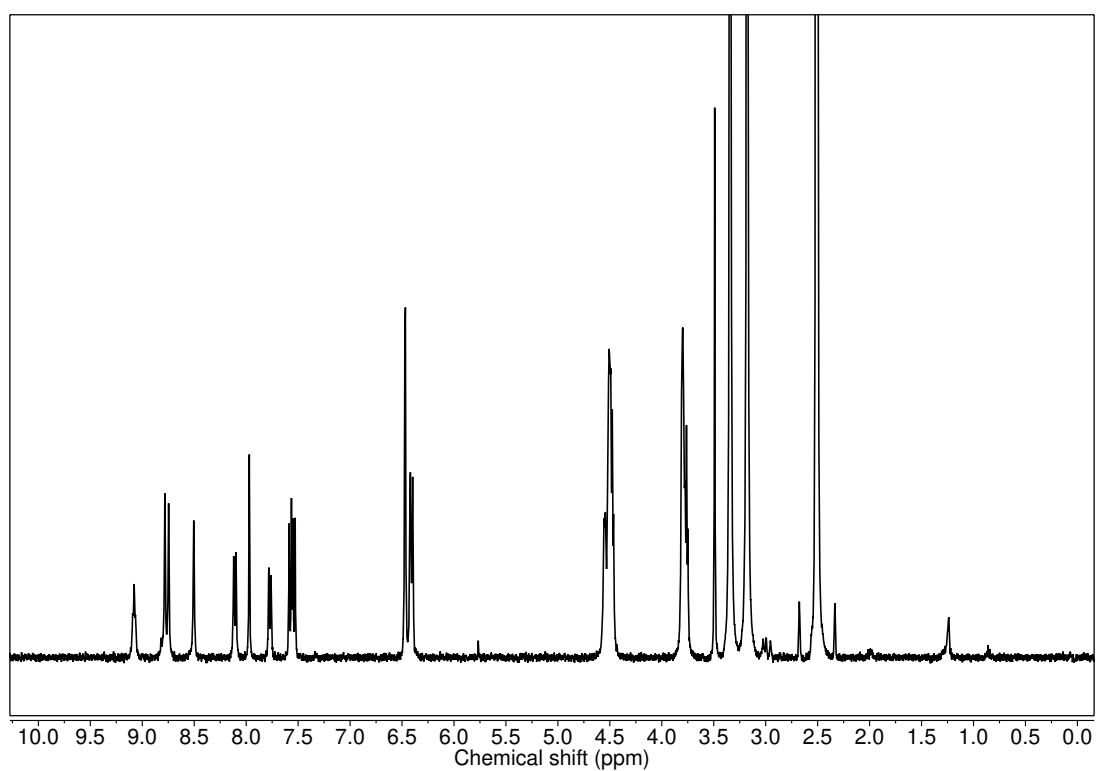


Figure A.8 ^1H NMR spectrum of compound **8** (DMSO-d_6).

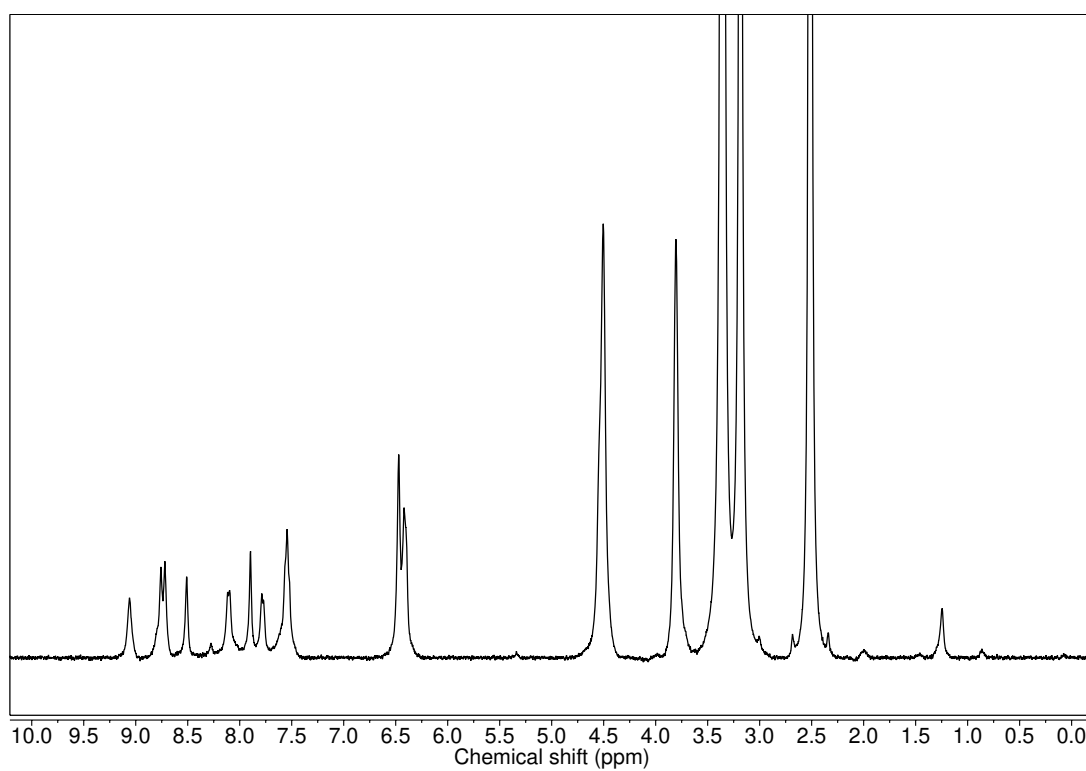


Figure A.9 ^1H NMR spectrum of compound **9** (DMSO-d_6).

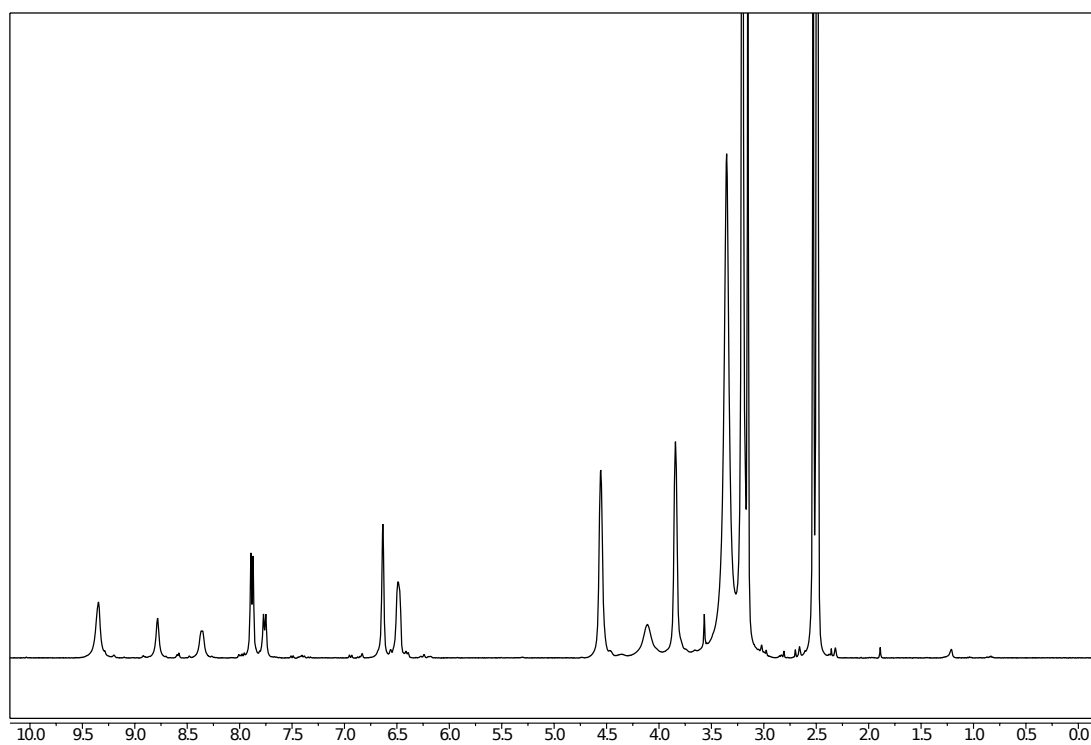


Figure A.10 ^1H NMR spectrum of compound 13 (DMSO-d_6).

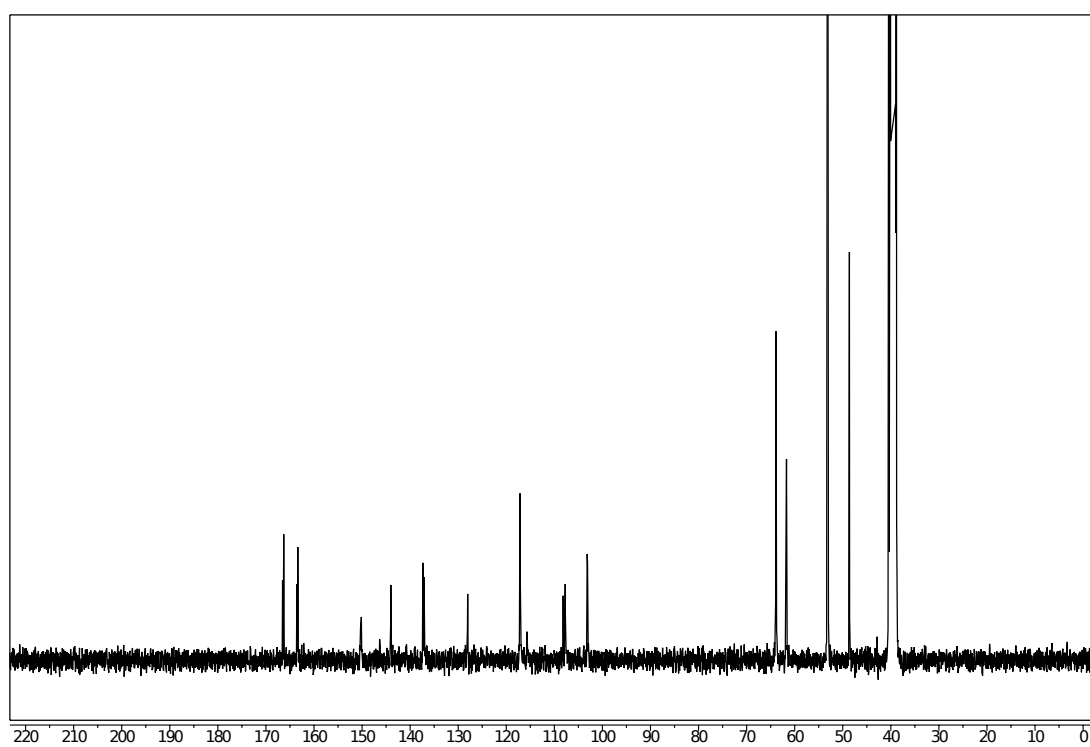


Figure A.11 ^{13}C NMR spectrum of compound 13 (DMSO-d_6).

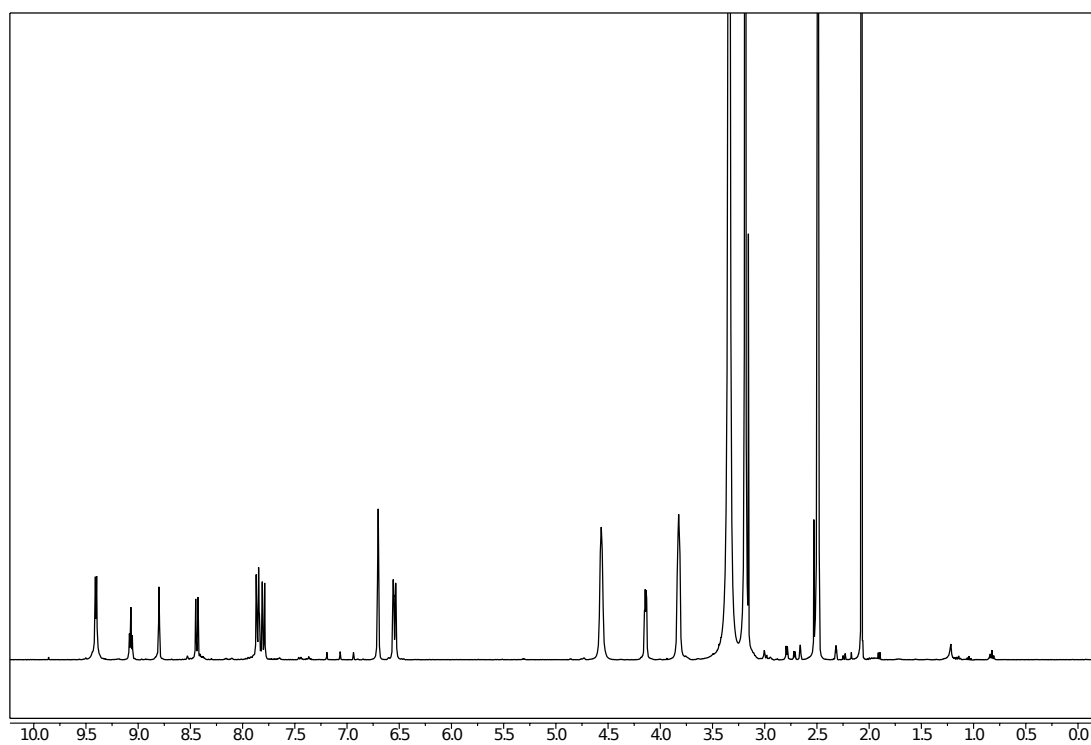


Figure A.12 ^1H NMR spectrum of compound **15** (DMSO-d_6).

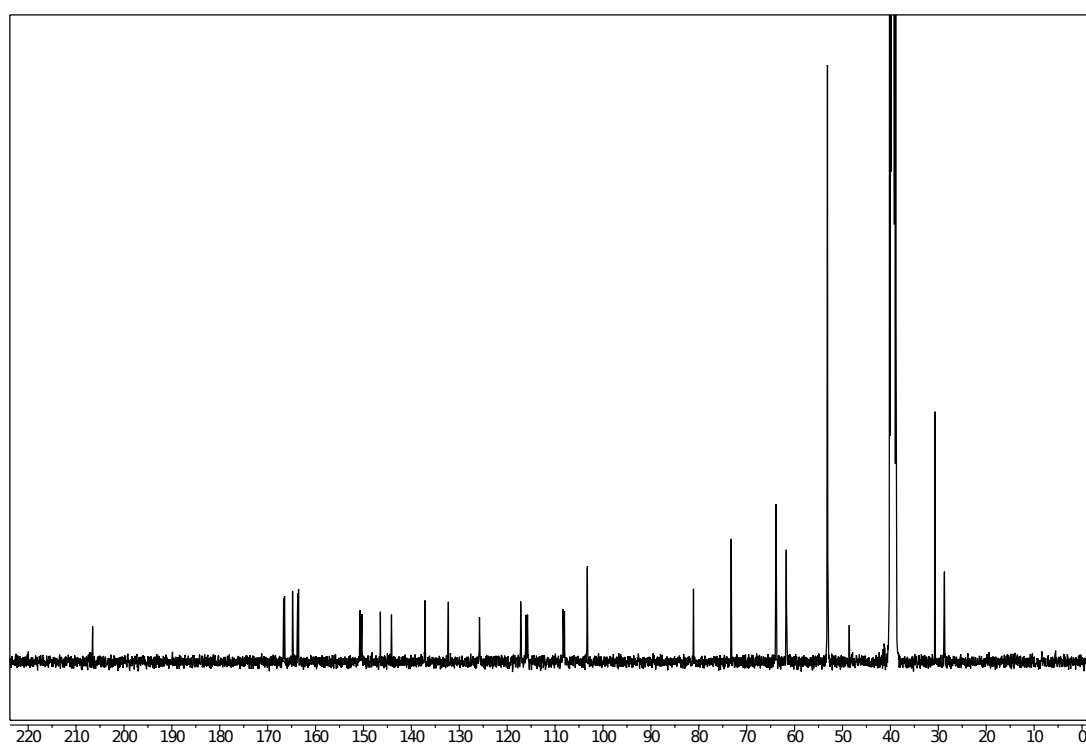


Figure A.13 ^{13}C NMR spectrum of compound **15** (DMSO-d_6).

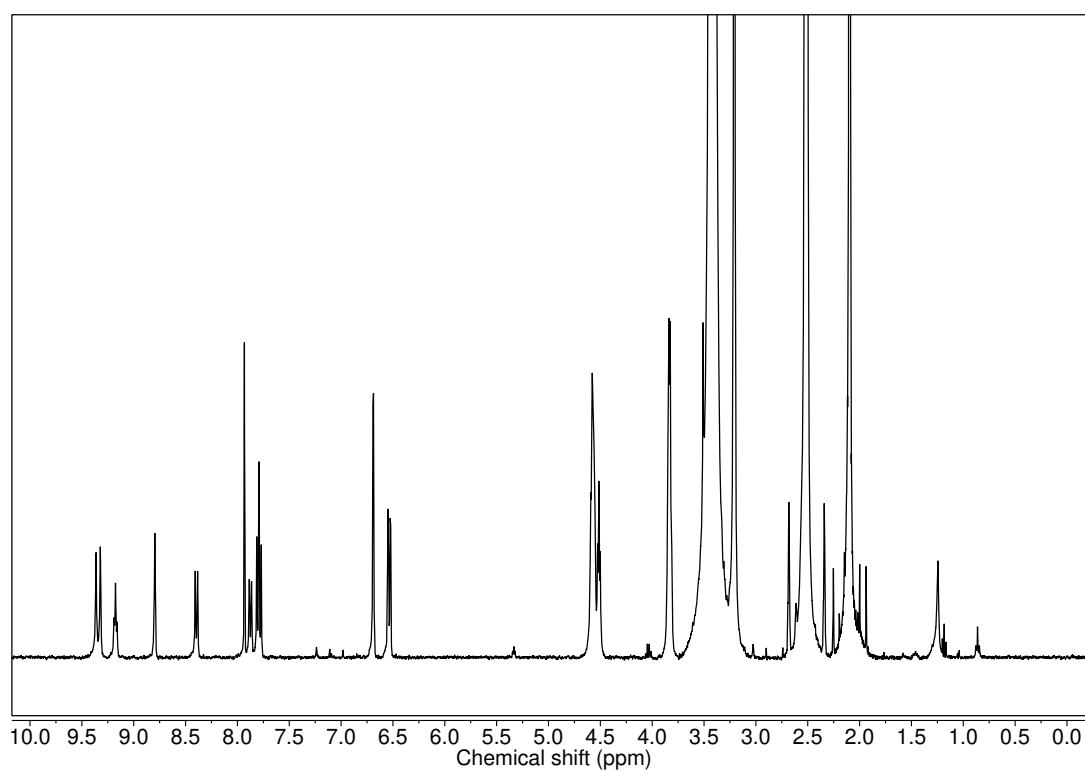


Figure A.14 ^1H NMR spectrum of compound **16** (DMSO-d_6).

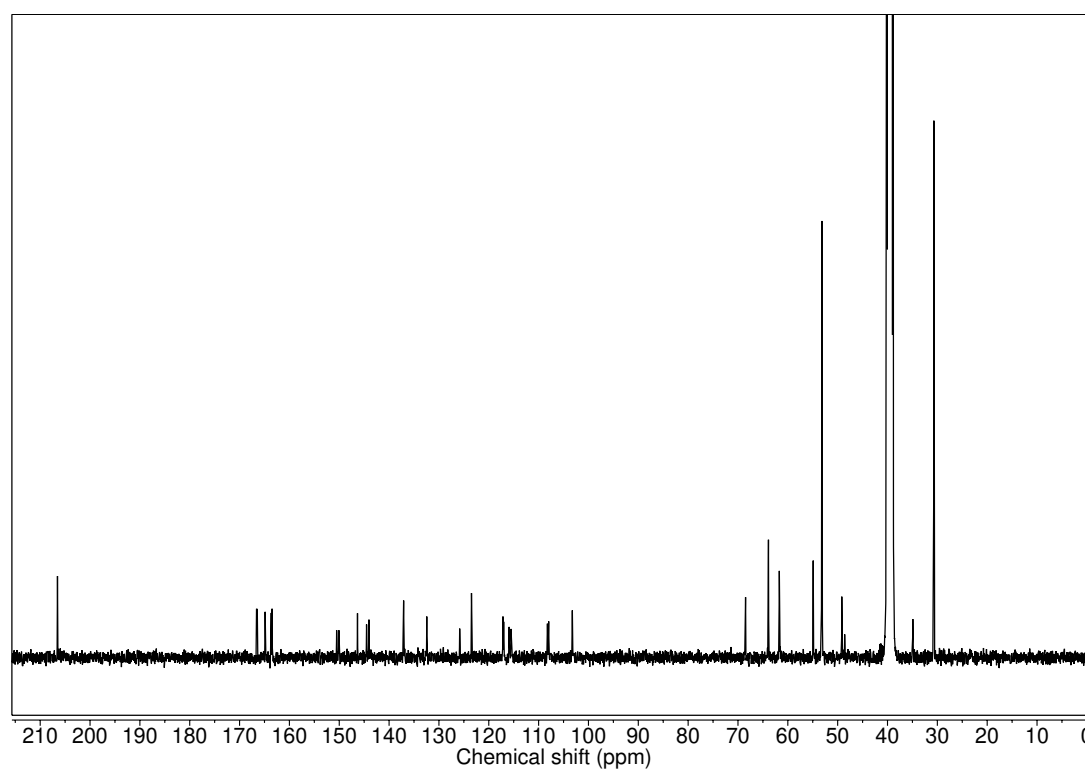


Figure A.15 ^{13}C NMR spectrum of compound **16** (DMSO-d_6).

A.1.2 Chapter 3

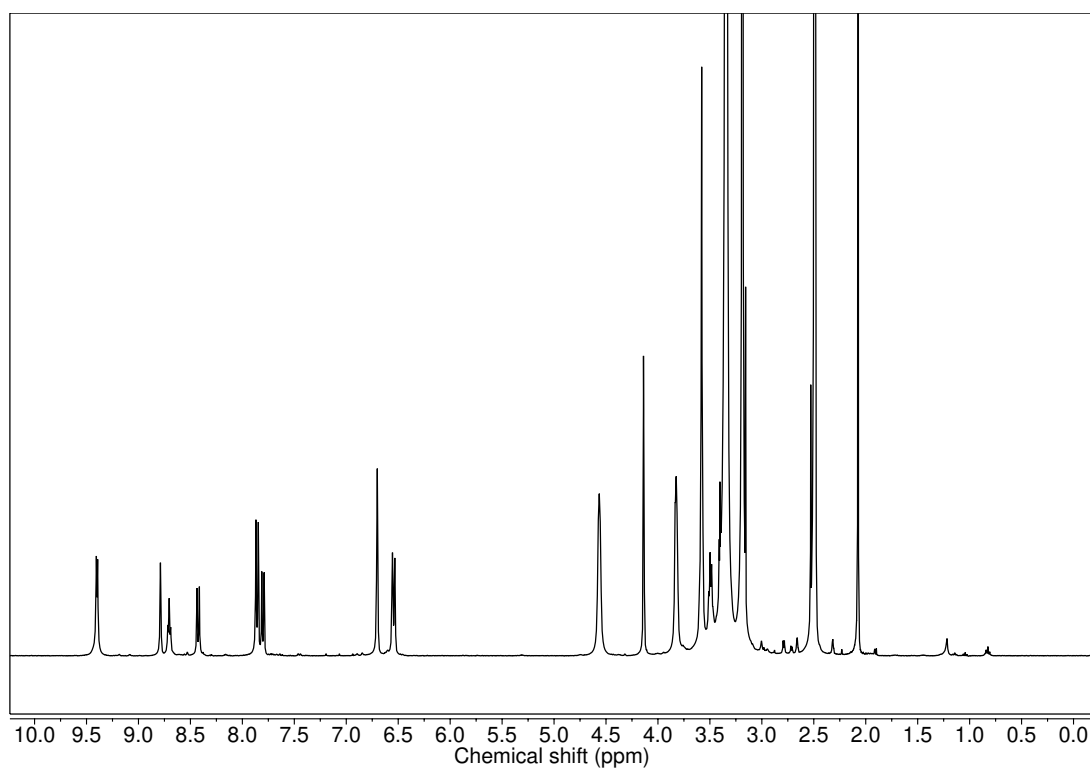


Figure A.16 ^1H NMR spectrum of compound 25 (DMSO-d_6).

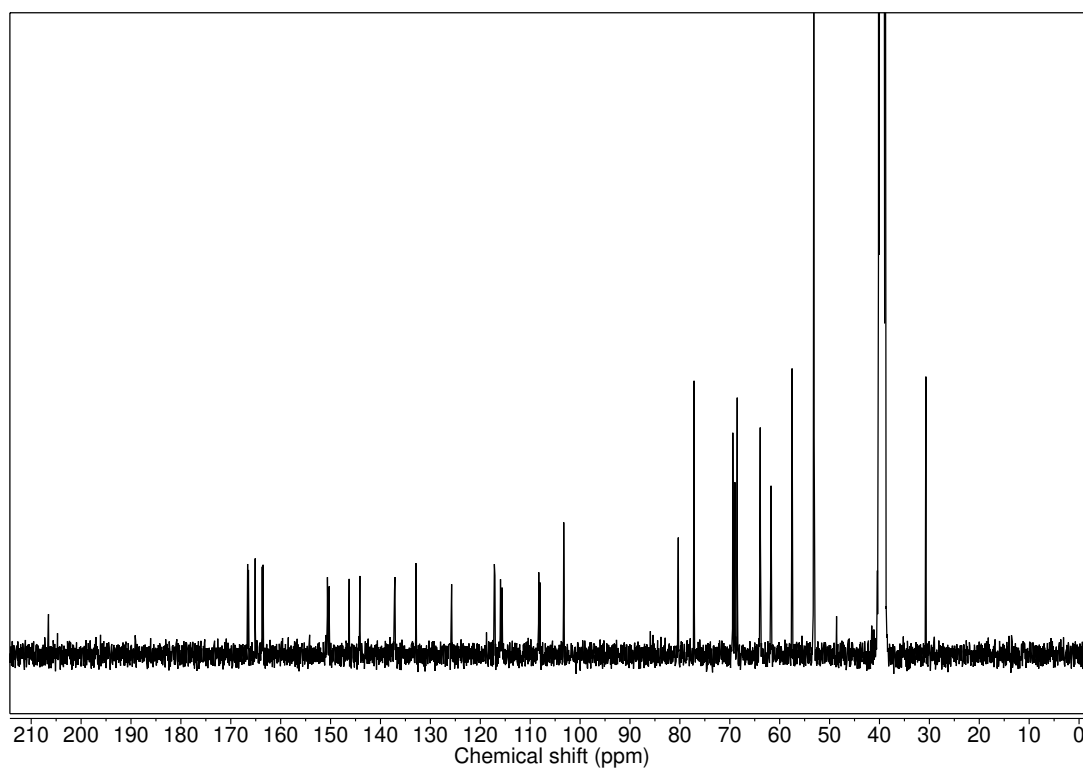


Figure A.17 ^{13}C NMR spectrum of compound 25 (DMSO-d_6).

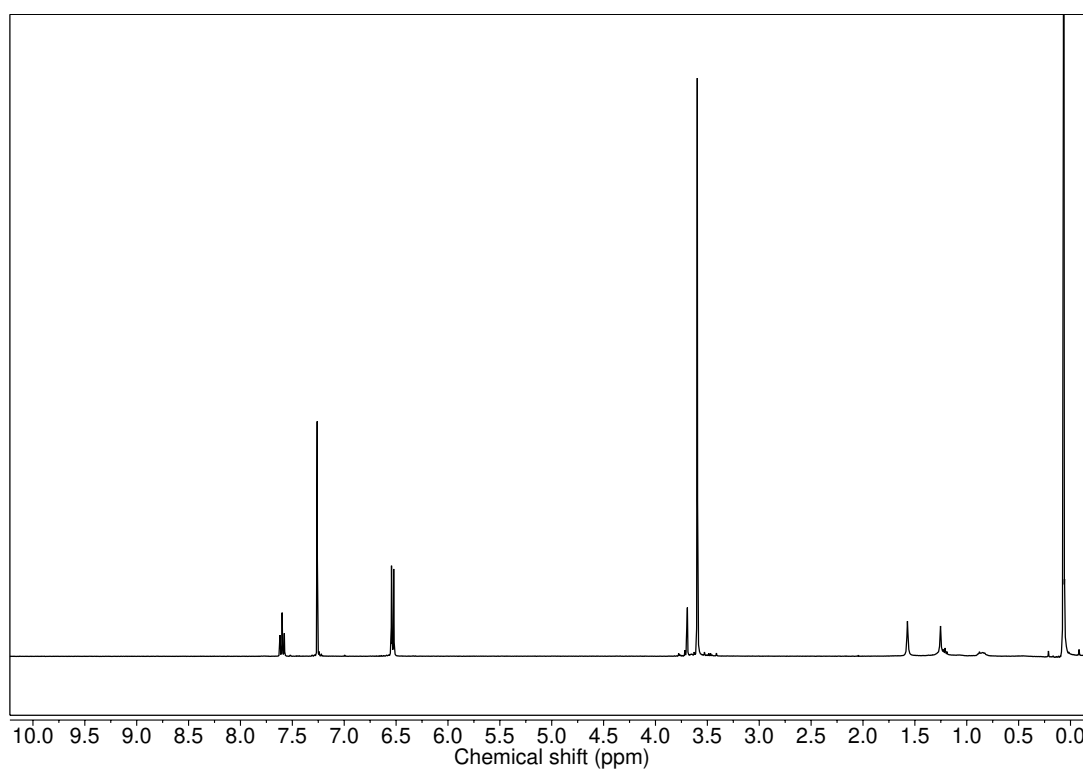


Figure A.18 ^1H NMR spectrum of compound 26 (CDCl_3).

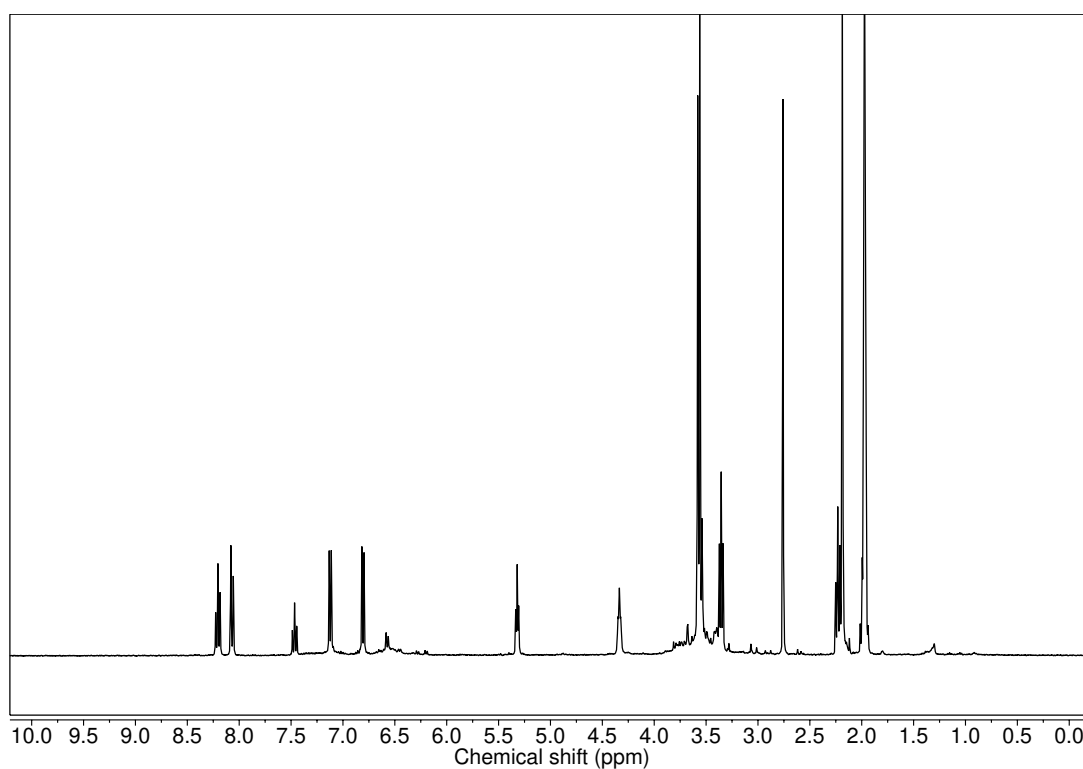


Figure A.19 ^1H NMR spectrum of compound 27 (CD_3CN).

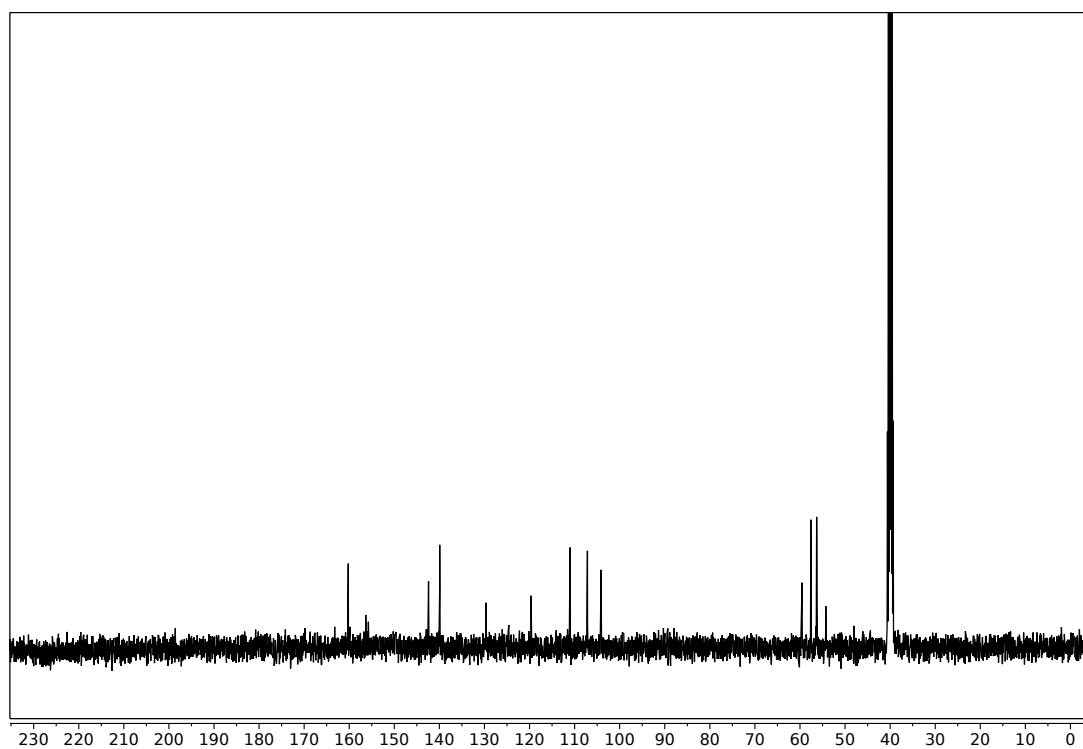


Figure A.20 ^{13}C NMR spectrum of compound 27 ($\text{DMSO}-d_6$).

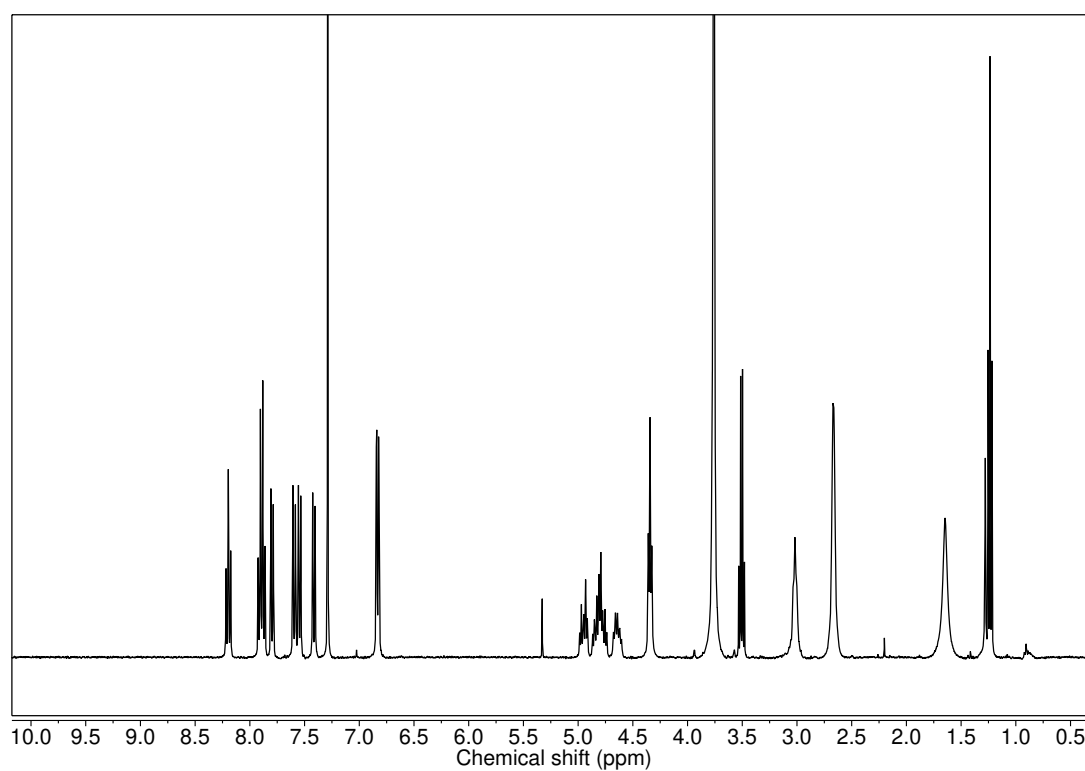


Figure A.21 ^1H NMR spectrum of compound 28 (CDCl_3).

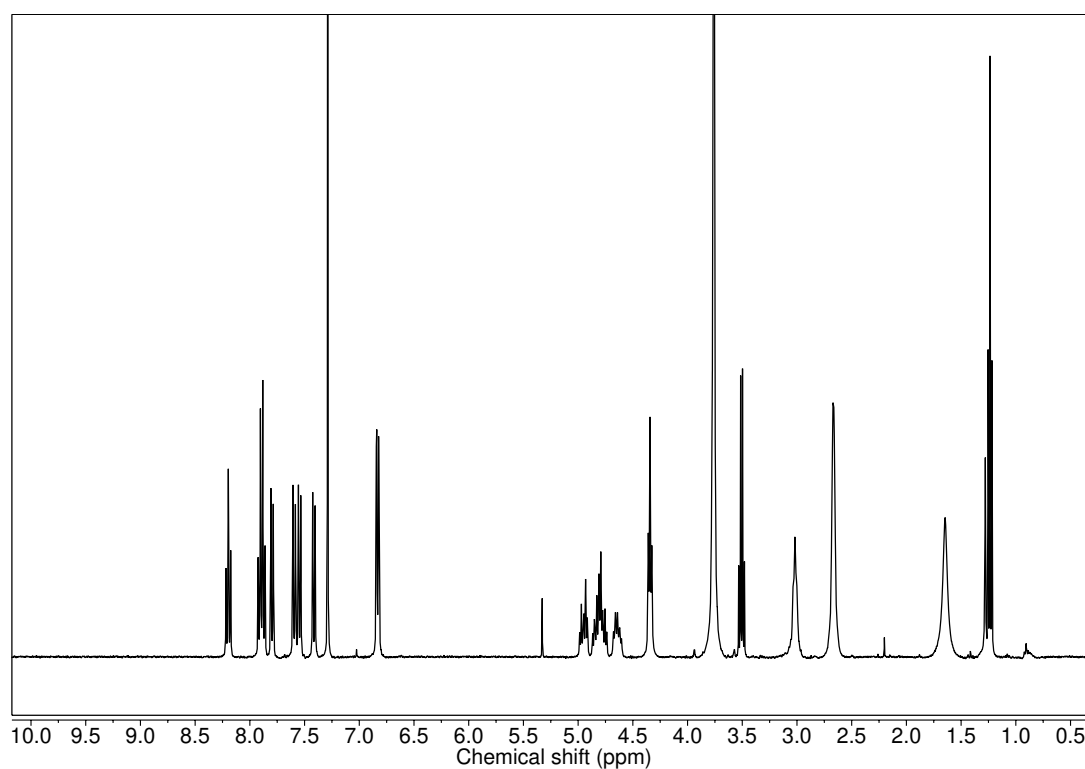


Figure A.22 ^{13}C NMR spectrum of compound 28 (DMSO-d_6).

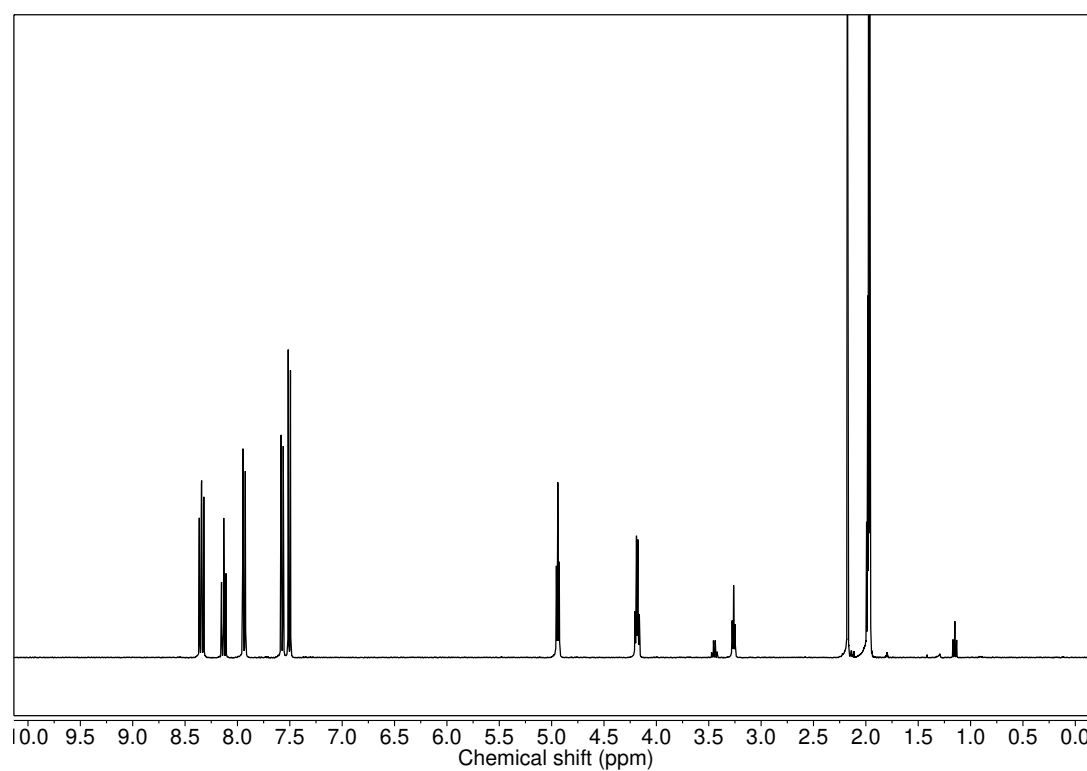


Figure A.23 ^1H NMR spectrum of compound 29 (CD_3CN).

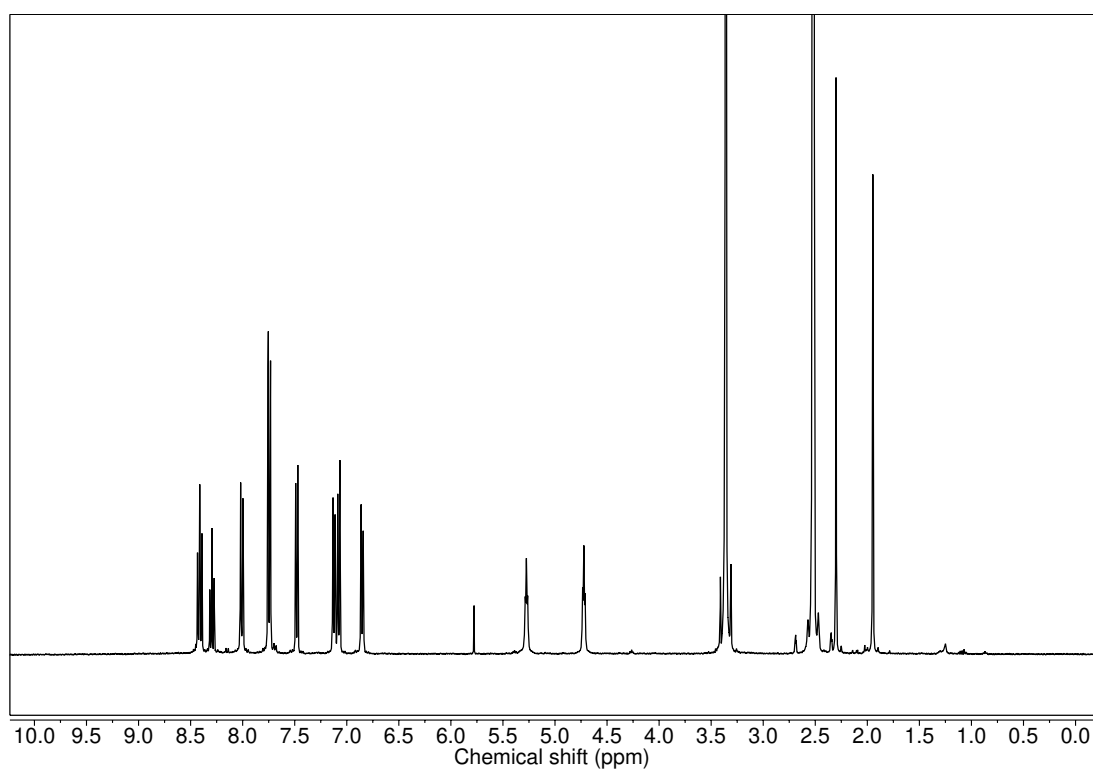


Figure A.24 ^1H NMR spectrum of compound 30 (CD_3CN).

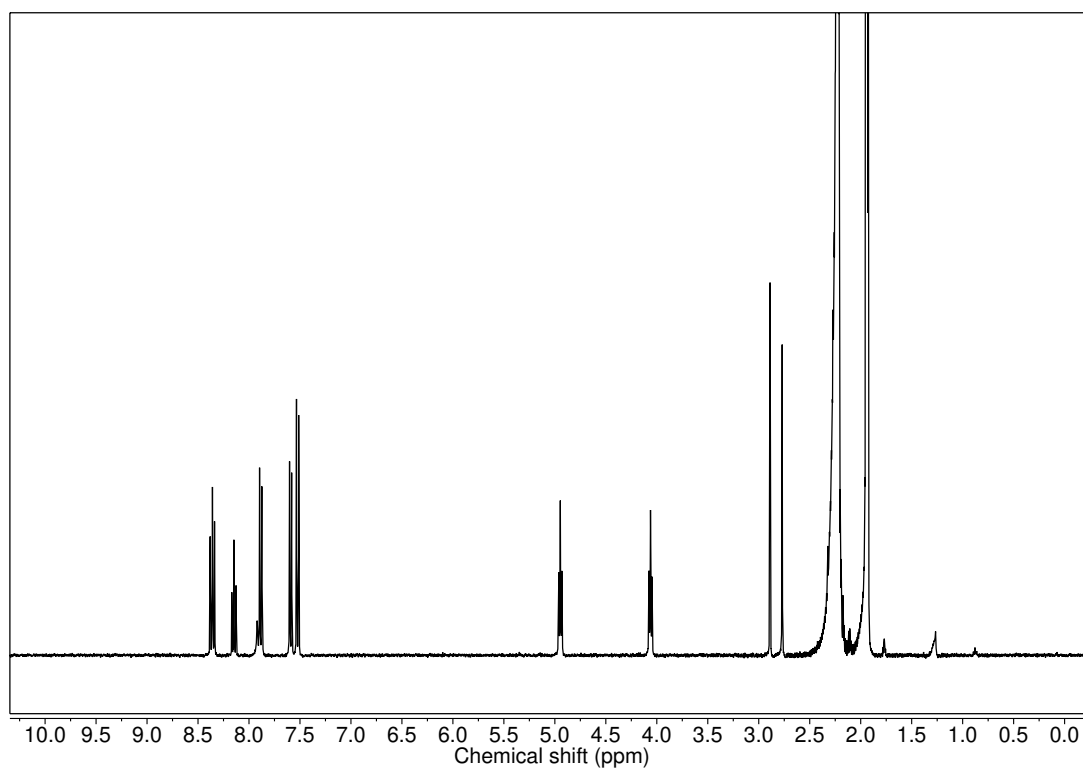


Figure A.25 ^1H NMR spectrum of compound **31** (CD_3CN).

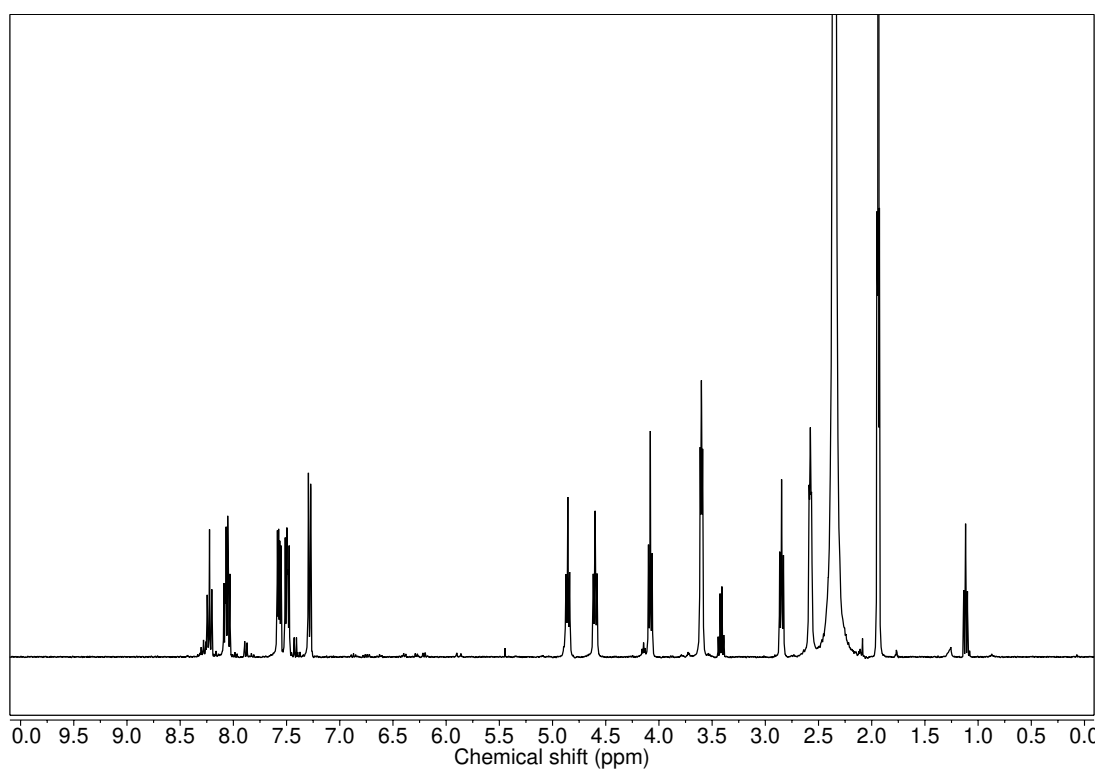


Figure A.26 ^1H NMR spectrum of compound **32** (CD_3CN).

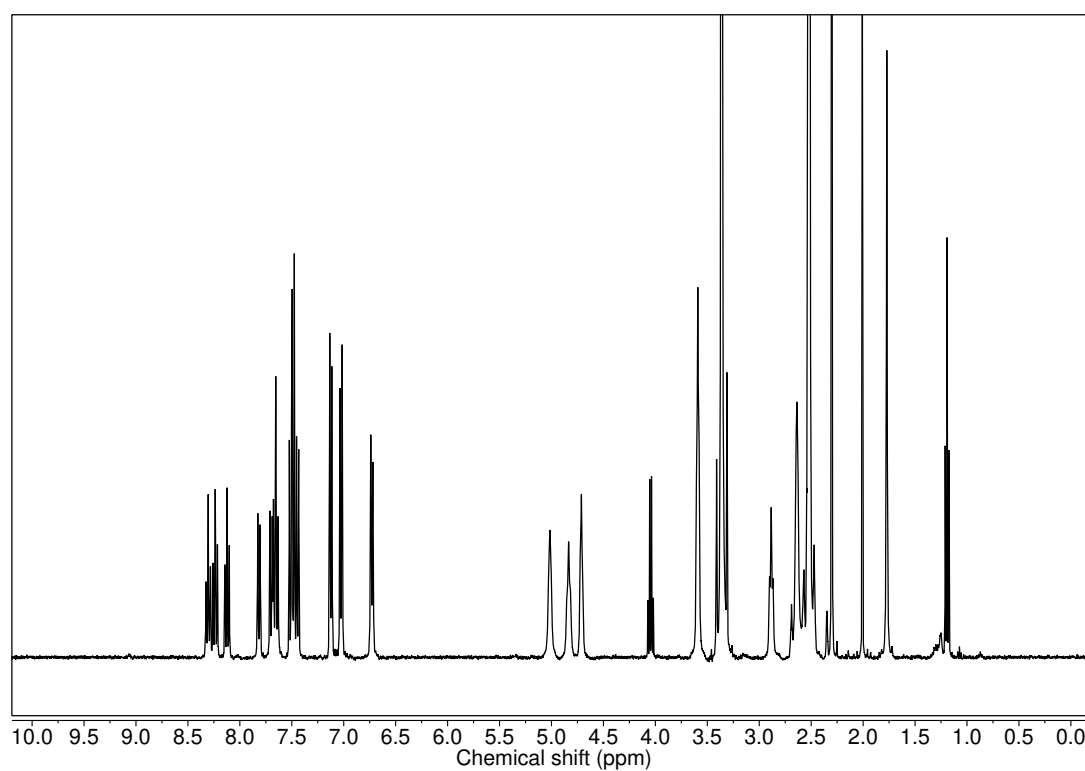


Figure A.27 ^1H NMR spectrum of compound 33 (DMSO-d_6).

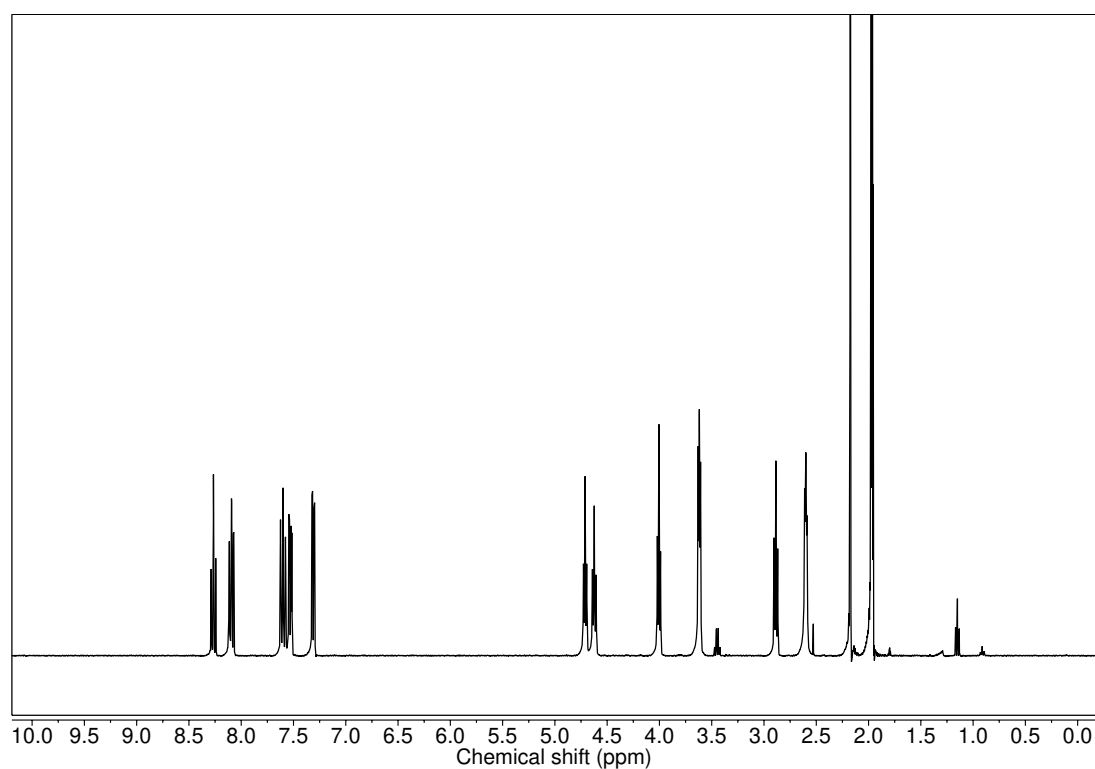


Figure A.28 ^1H NMR spectrum of compound 34 (CD_3CN).

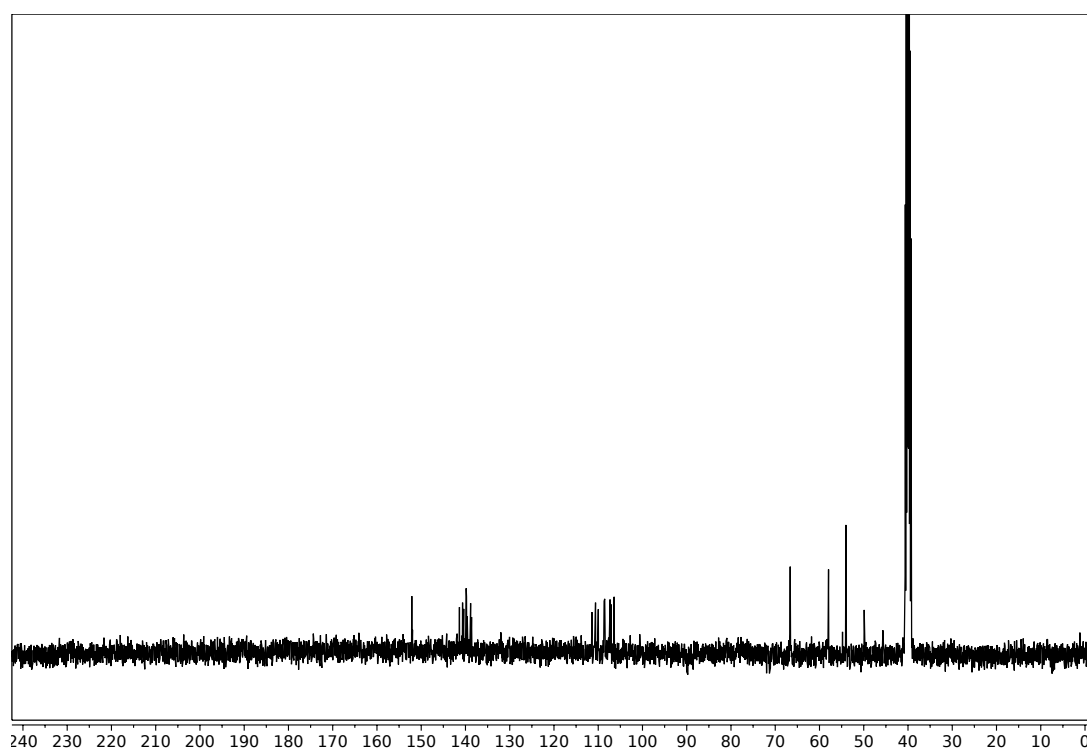


Figure A.29 ^{13}C NMR spectrum of compound 34 (DMSO-d_6).

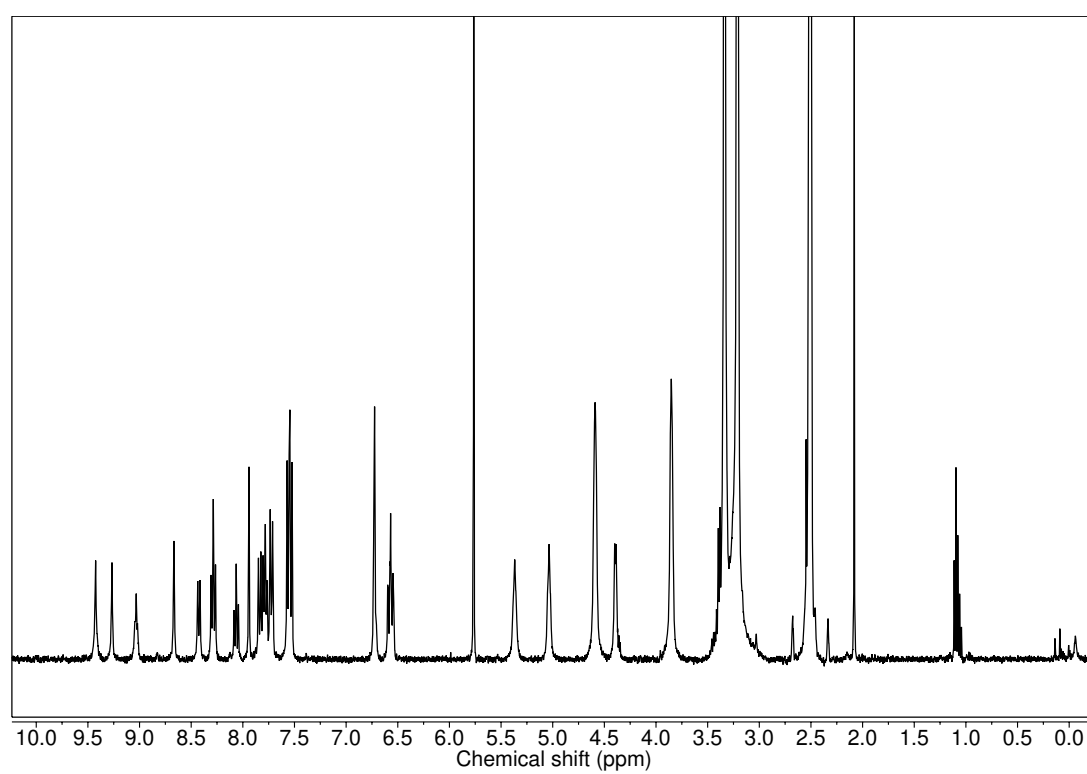


Figure A.30 ^1H NMR spectrum of compound 35 (DMSO-d_6).

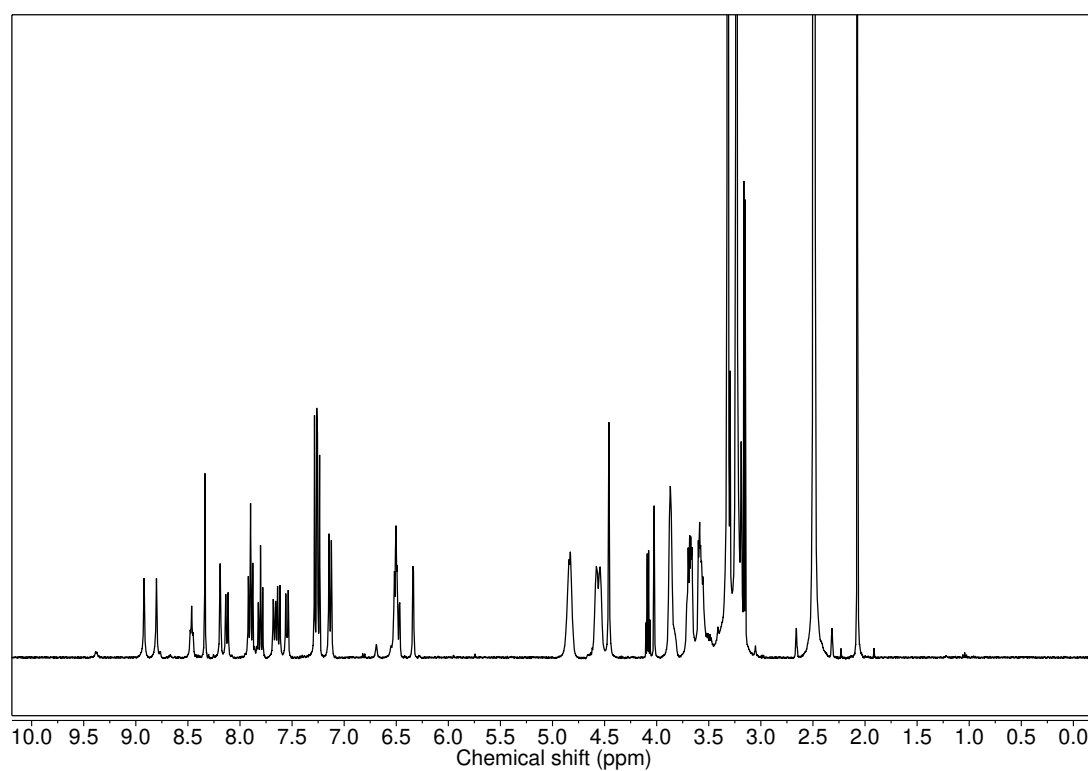


Figure A.31 ^1H NMR spectrum of compound **36** (DMSO-d_6).

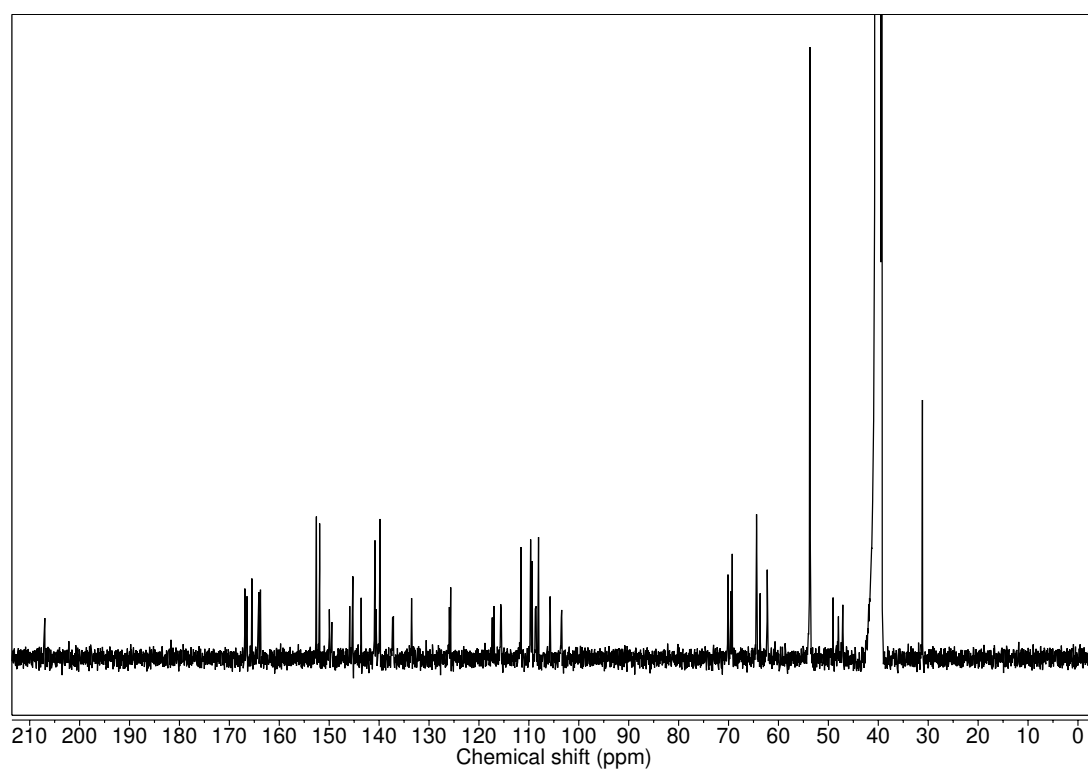


Figure A.32 ^{13}C NMR spectrum of compound **36** (DMSO-d_6).

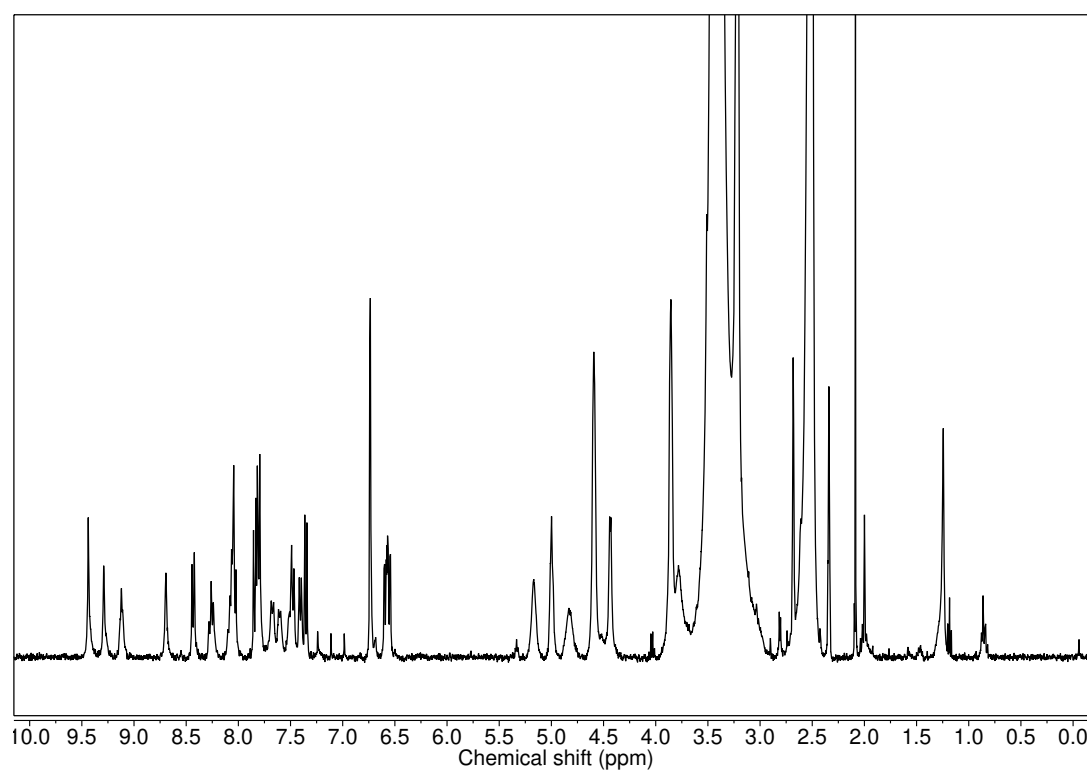


Figure A.33 ^1H NMR spectrum of compound **37** (DMSO-d_6).

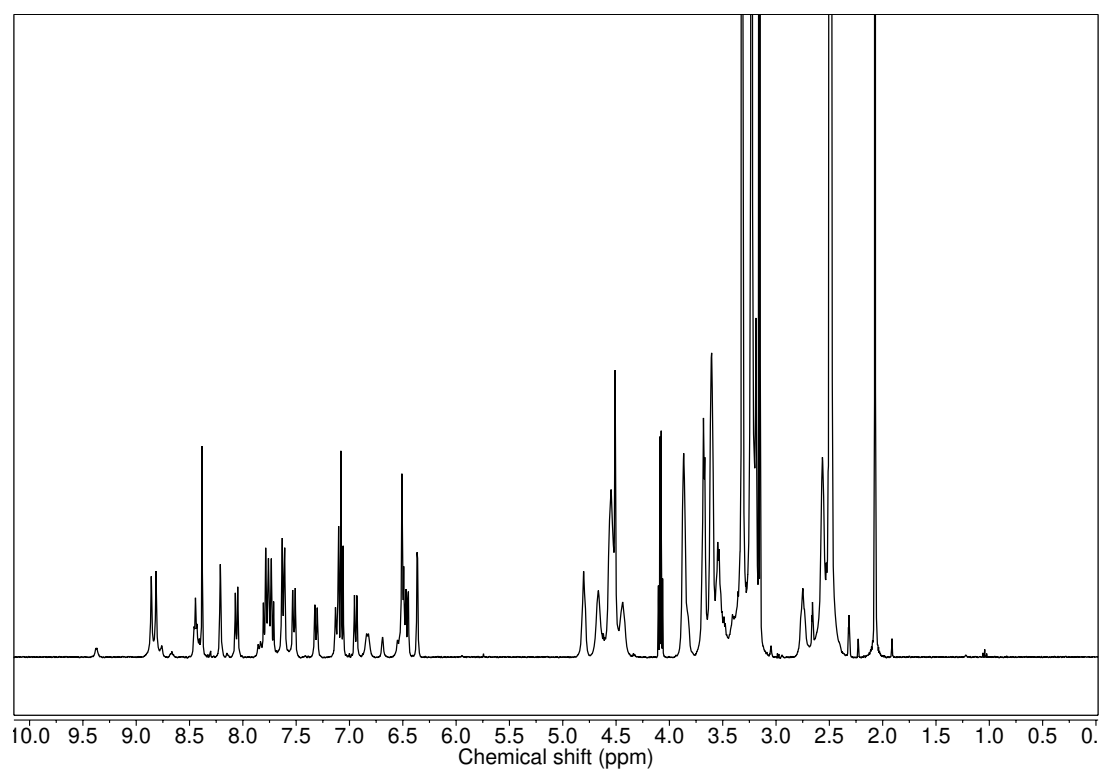


Figure A.34 ^1H NMR spectrum of compound **38** (DMSO-d_6).

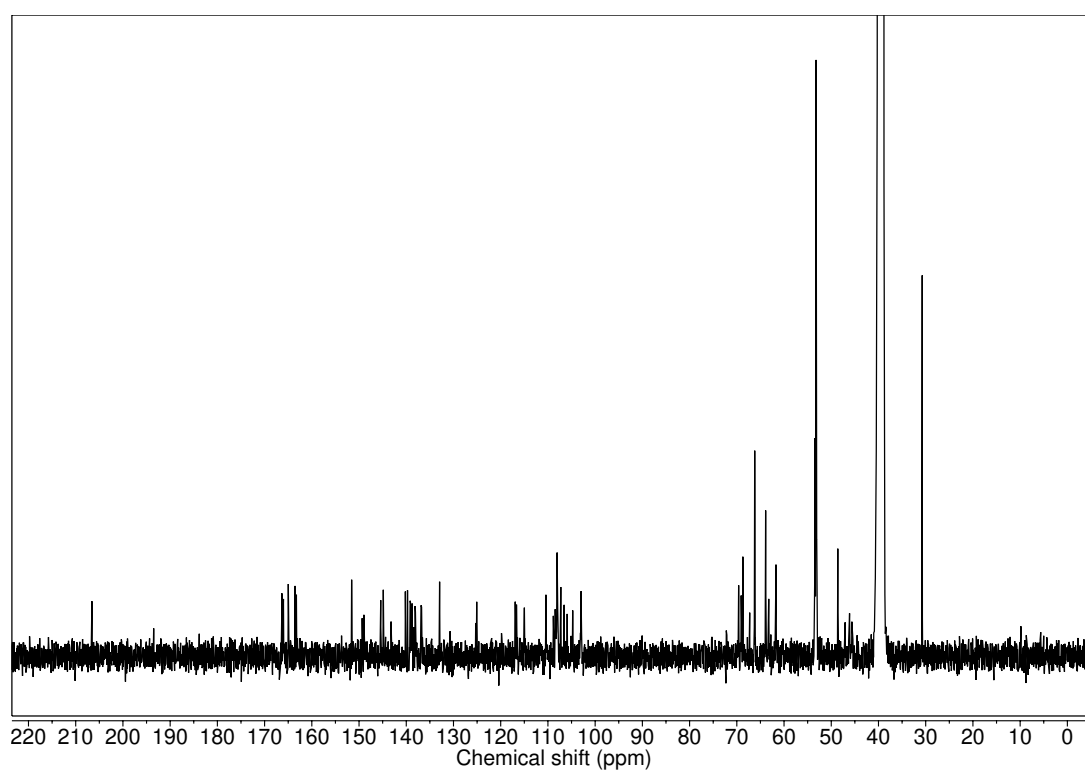


Figure A.35 ^{13}C NMR spectrum of compound **38** (DMSO- d_6).

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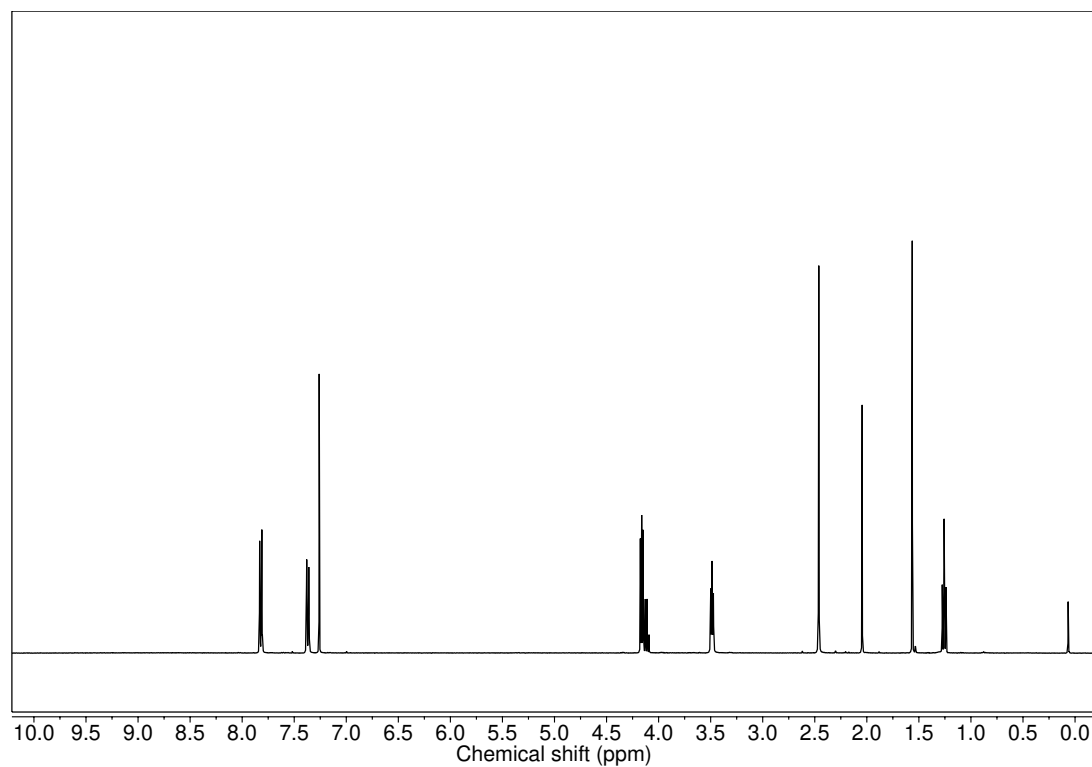


Figure A.36 ^1H NMR spectrum of compound 39 (CDCl_3).

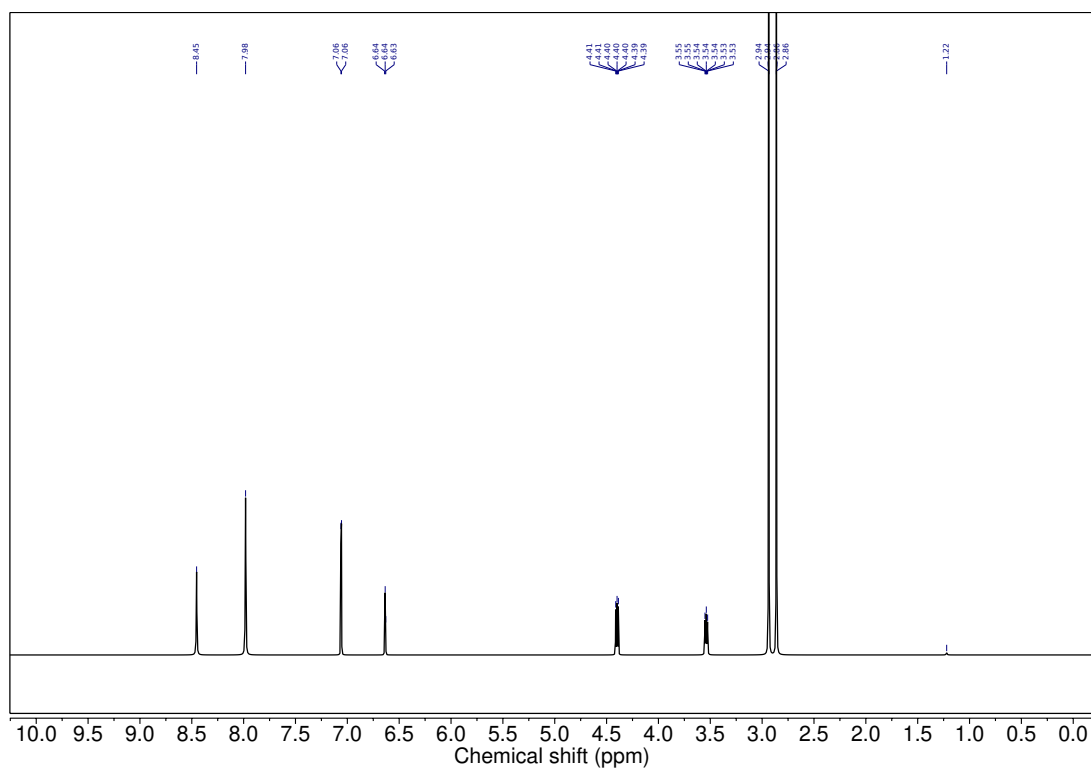


Figure A.37 ^1H NMR spectrum of compound 40 (CDCl_3).

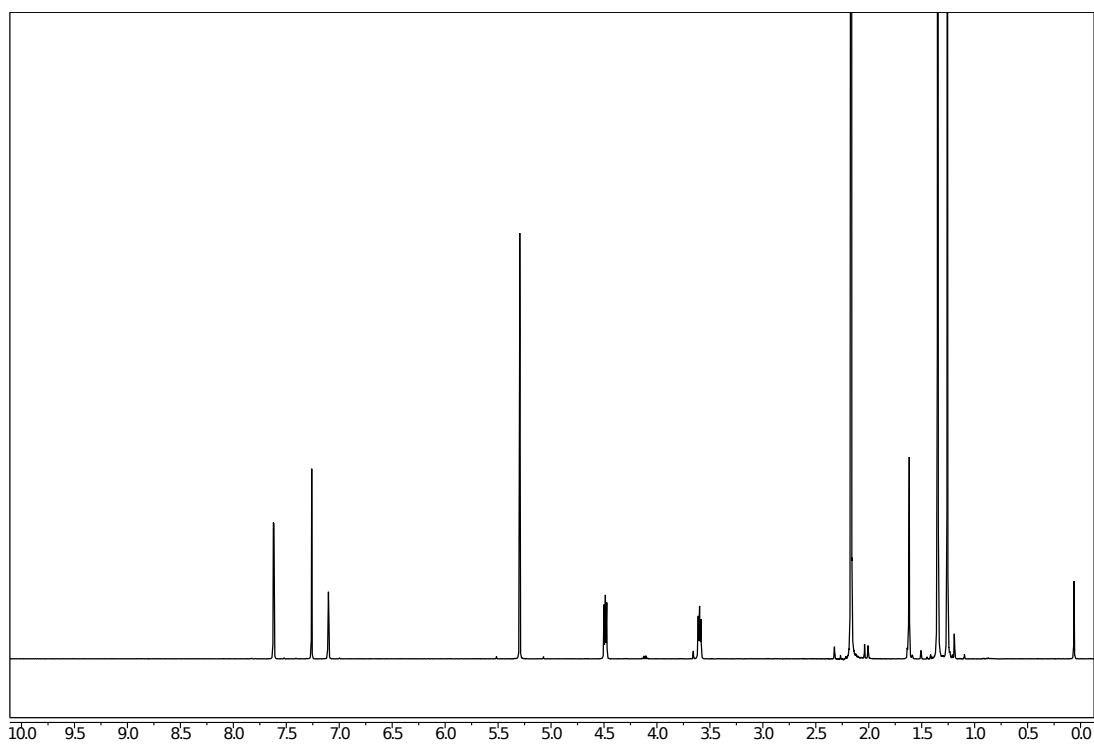


Figure A.38 ^1H NMR spectrum of compound 41 (CDCl_3).

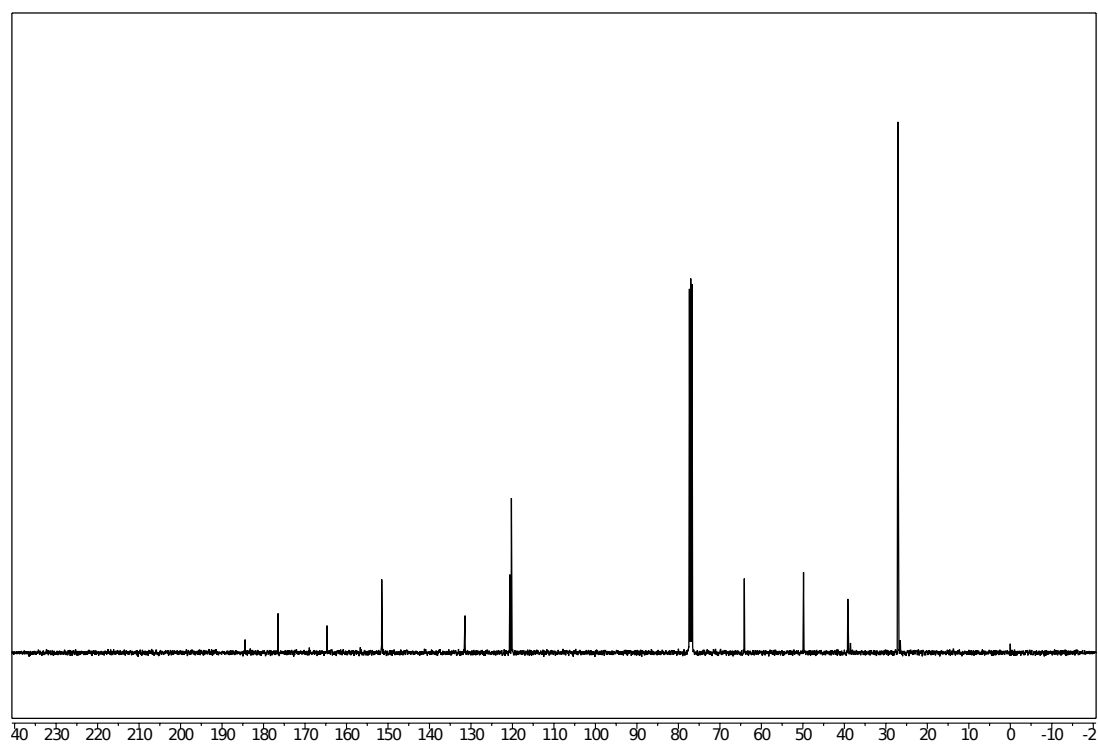


Figure A.39 ^{13}C NMR spectrum of compound 41 (CDCl_3).

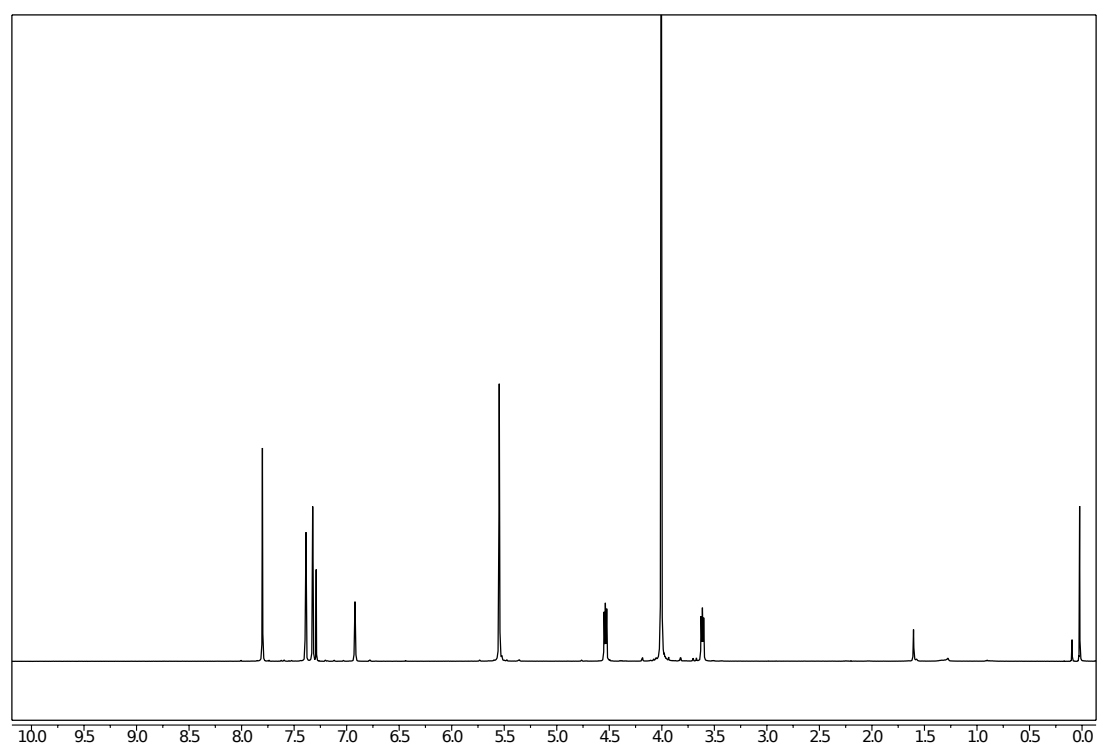


Figure A.40 ^1H NMR spectrum of compound 42 (CDCl_3).

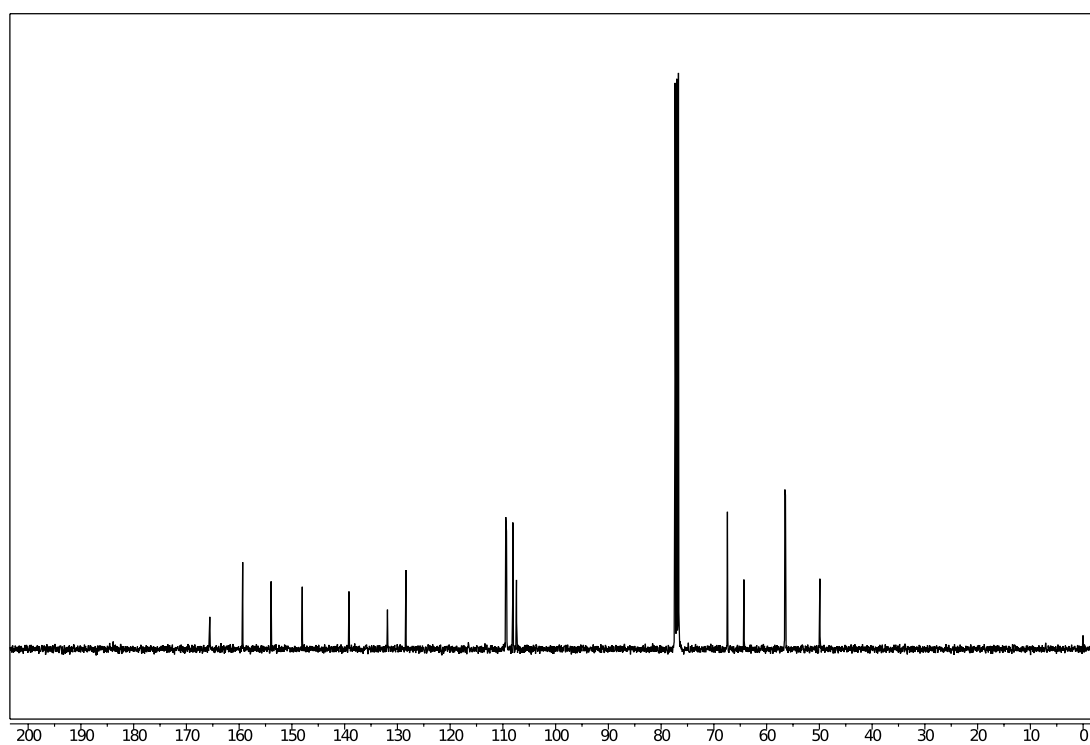


Figure A.41 ^{13}C NMR spectrum of compound 42 (CDCl_3).

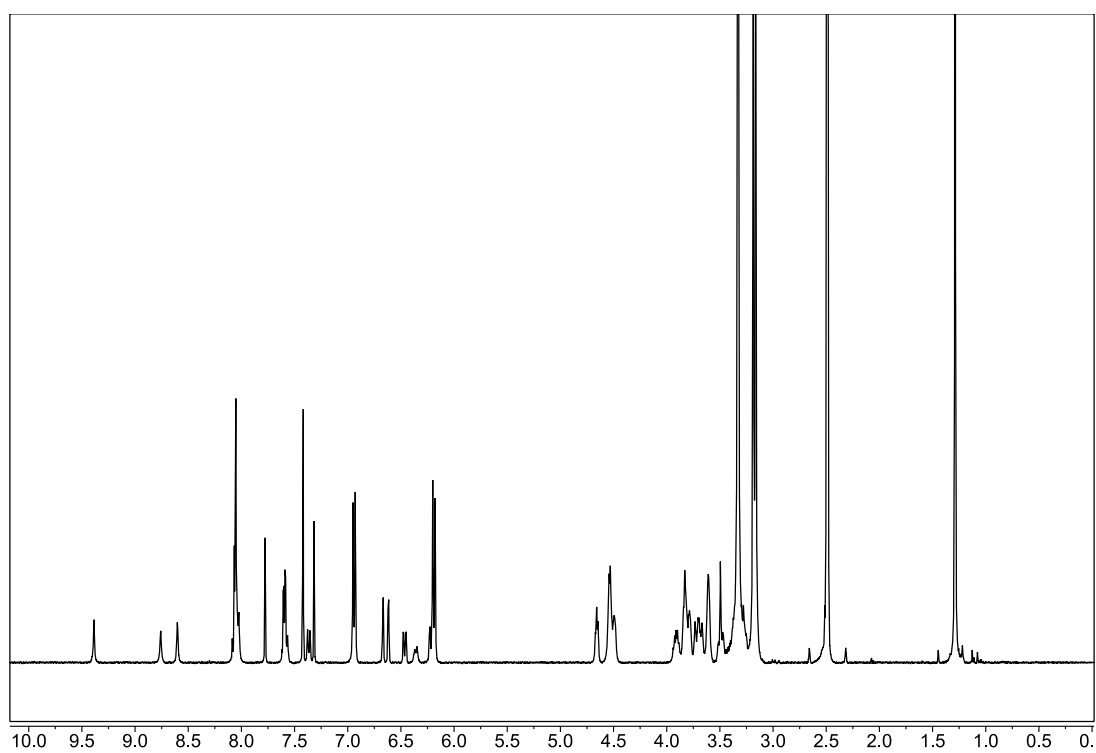


Figure A.42 ^1H NMR spectrum of compound 46^{enz} (DMSO-d_6).

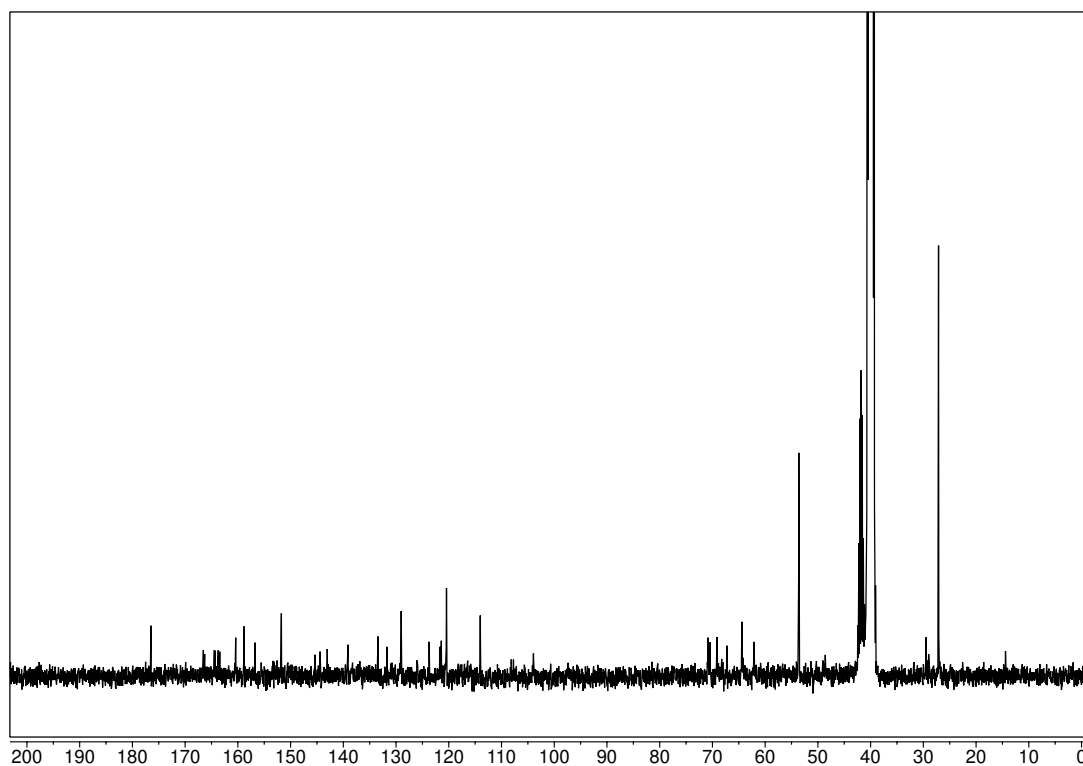


Figure A.43 ^{13}C NMR spectrum of compound **46**^{enz} (DMSO- d_6).

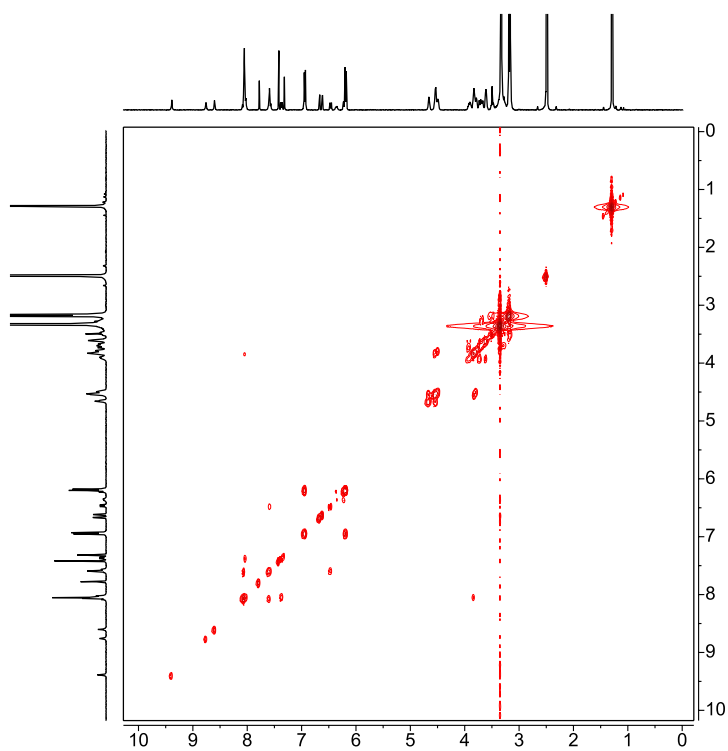


Figure A.44 COSY NMR spectrum of compound **46**^{enz} (DMSO- d_6).

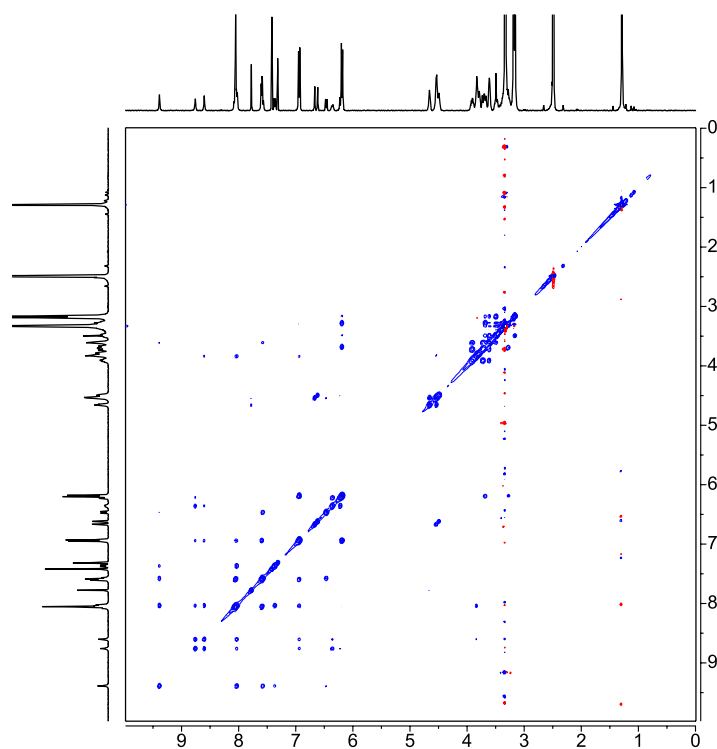


Figure A.45 NOESY NMR spectrum of compound 46^{enz} (DMSO-d₆).

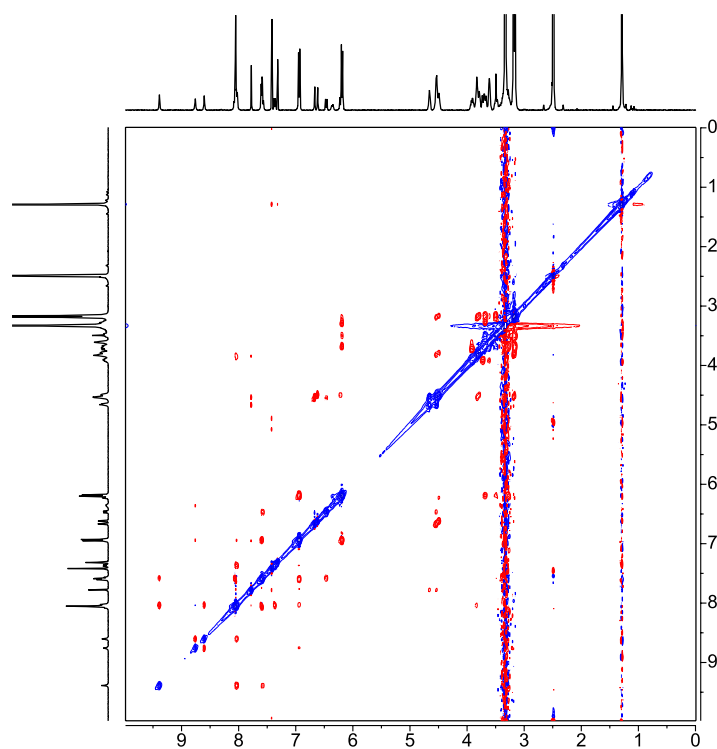


Figure A.46 ROESY NMR spectrum of compound 46^{enz} (DMSO-d₆).

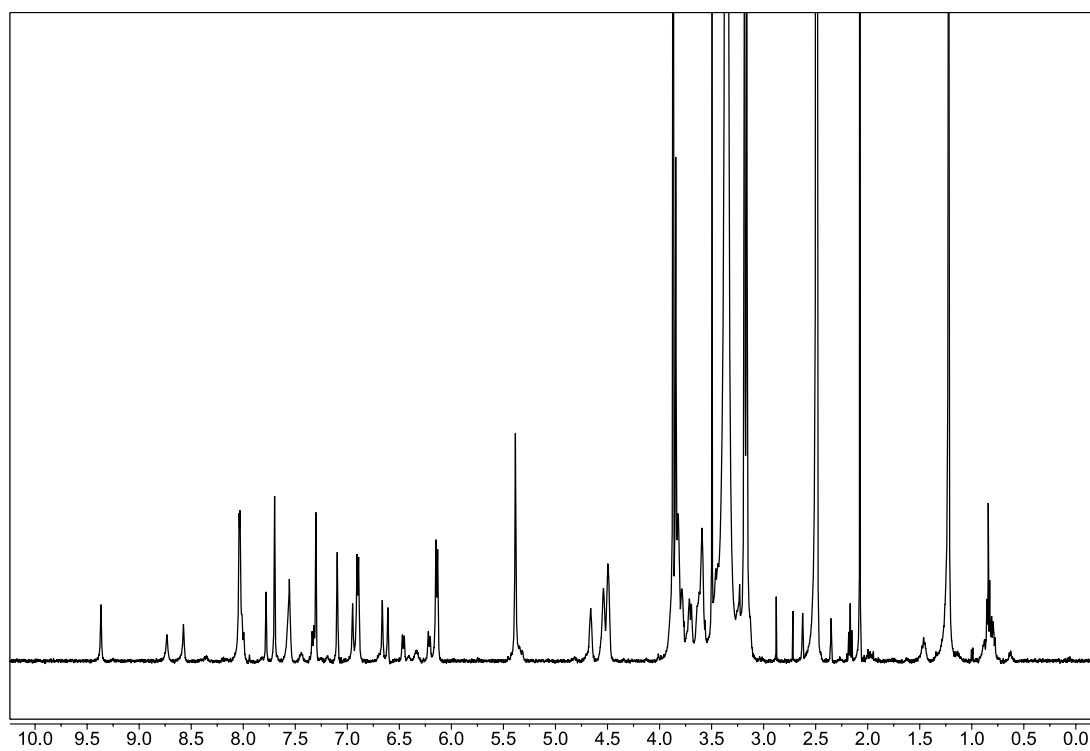


Figure A.47 ^1H NMR spectrum of compound **46**^{photo} (DMSO-d_6).

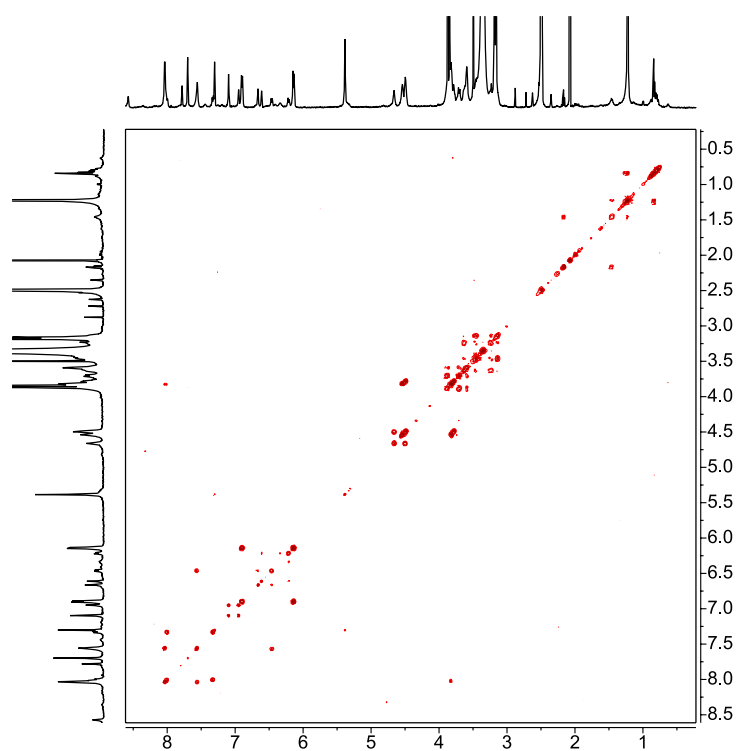


Figure A.48 COSY NMR spectrum of compound **46**^{photo} (DMSO-d_6).

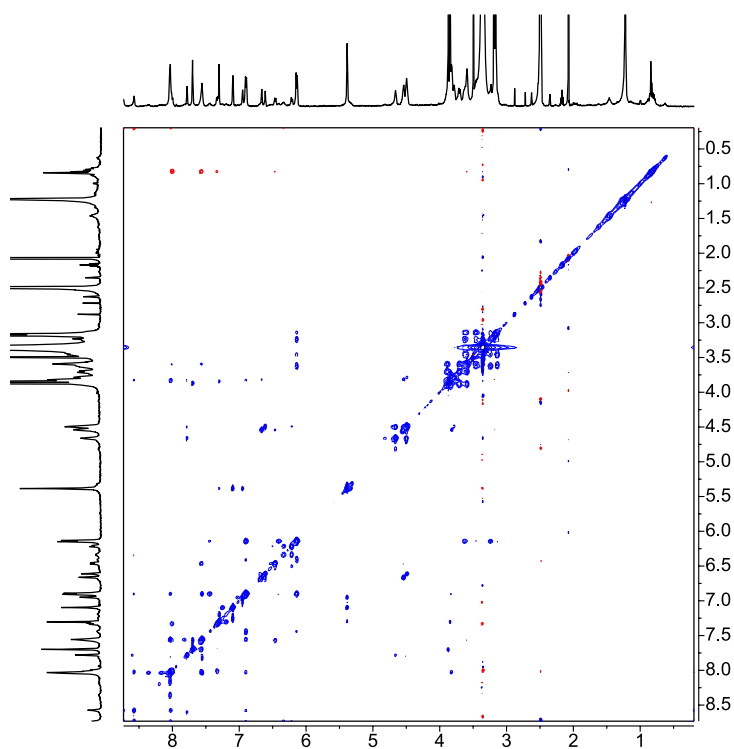


Figure A.49 NOESY NMR spectrum of compound **46**^{photo} (DMSO-d₆).

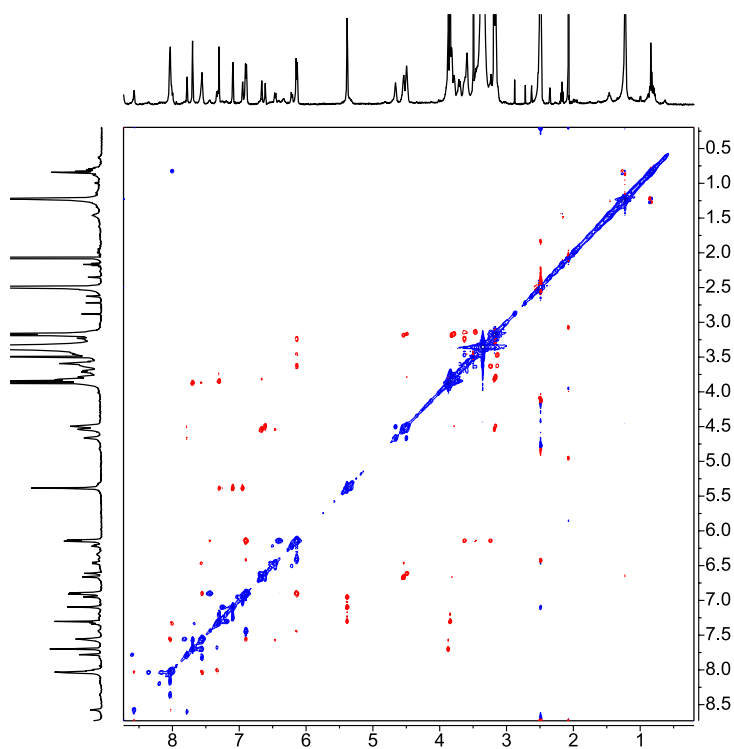


Figure A.50 ROESY NMR spectrum of compound **46**^{photo} (DMSO-d₆).

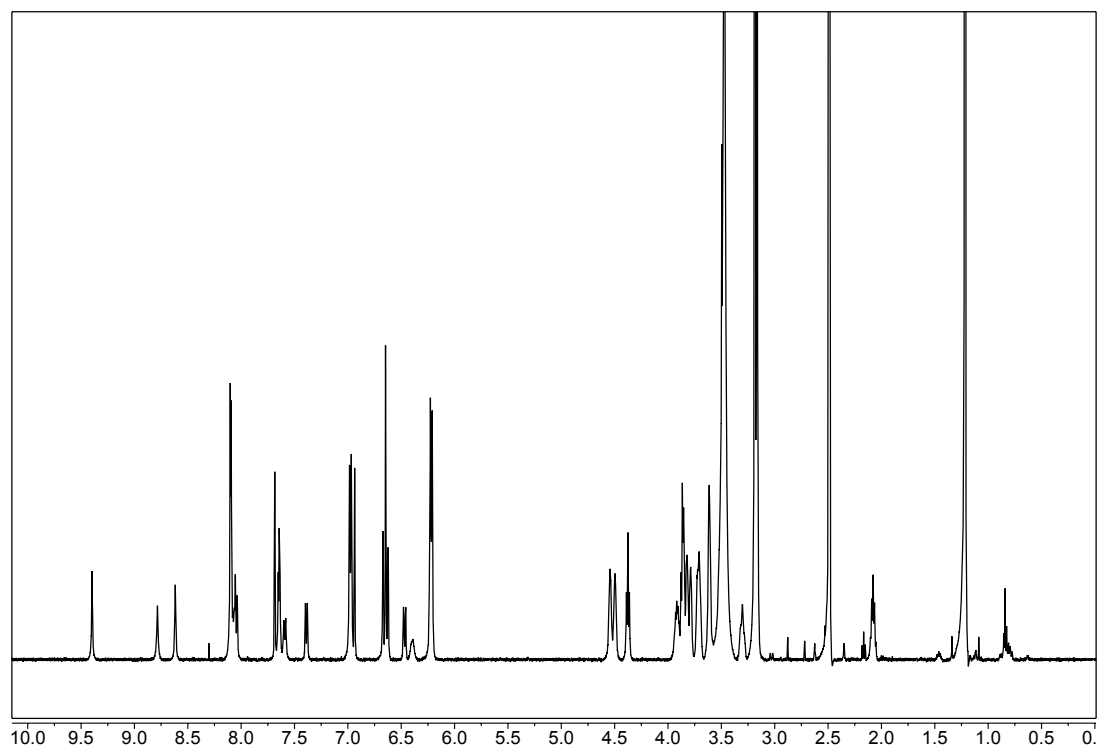


Figure A.51 ^1H NMR spectrum of compound **46^{ctrl}** (DMSO-d_6).

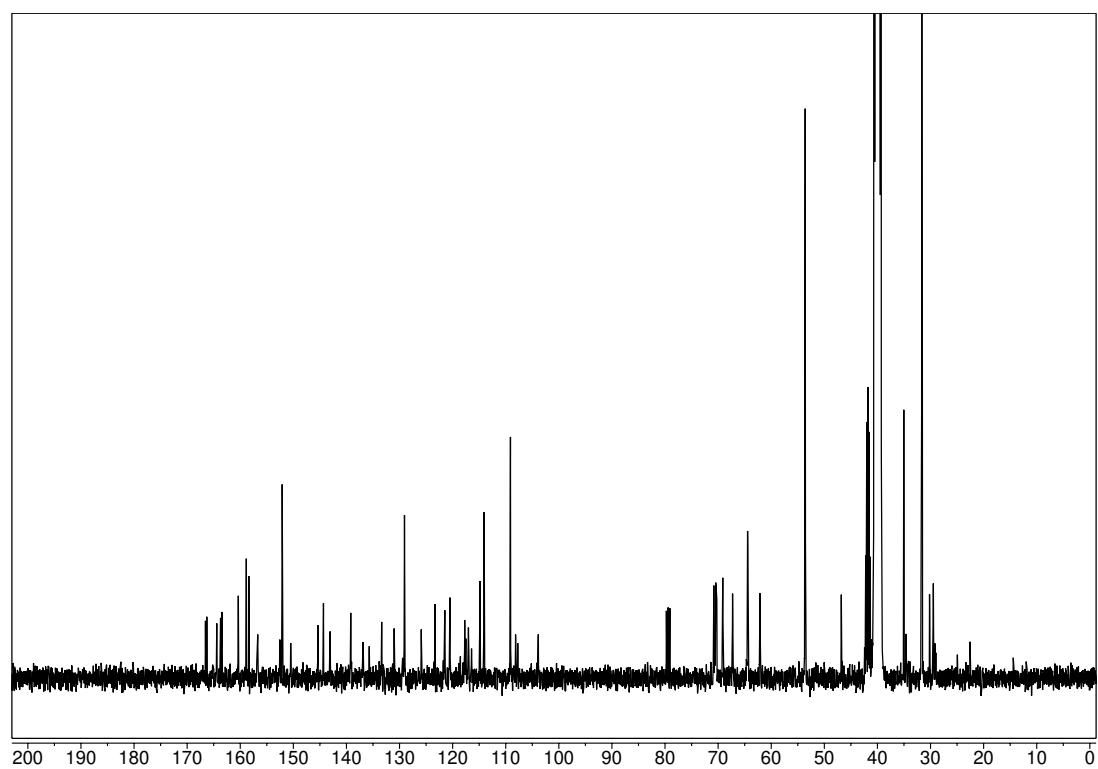


Figure A.52 ^{13}C NMR spectrum of compound **46^{ctrl}** (DMSO-d_6).

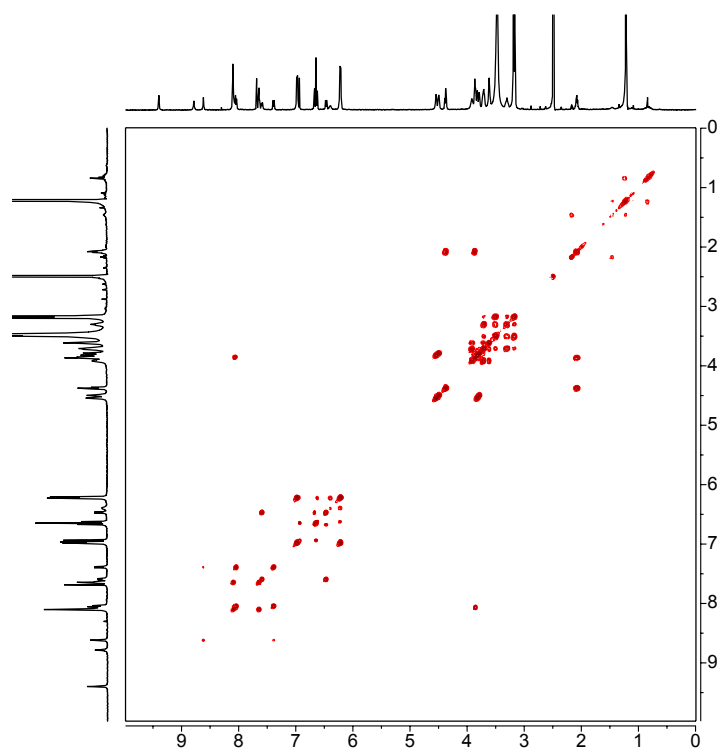


Figure A.53 COSY NMR spectrum of compound **46^{ctrl}** (DMSO-d₆).

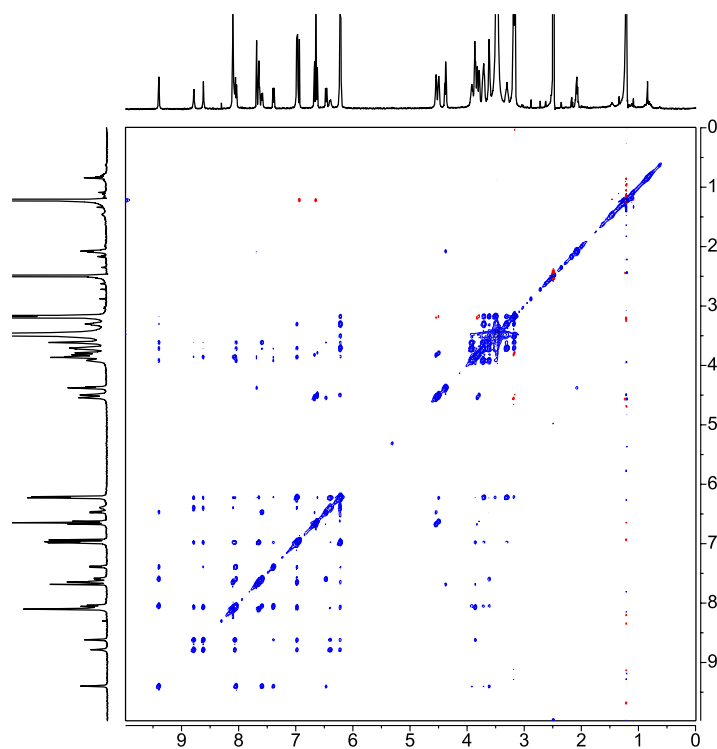


Figure A.54 NOESY NMR spectrum of compound **46^{ctrl}** (DMSO-d₆).

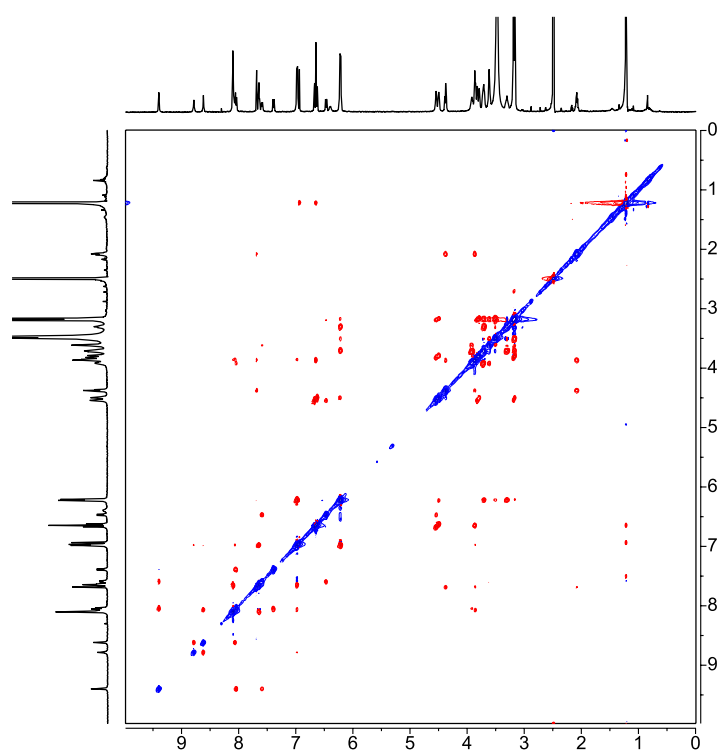


Figure A.55 ROESY NMR spectrum of compound **46^{ctrl}** (DMSO- d_6).

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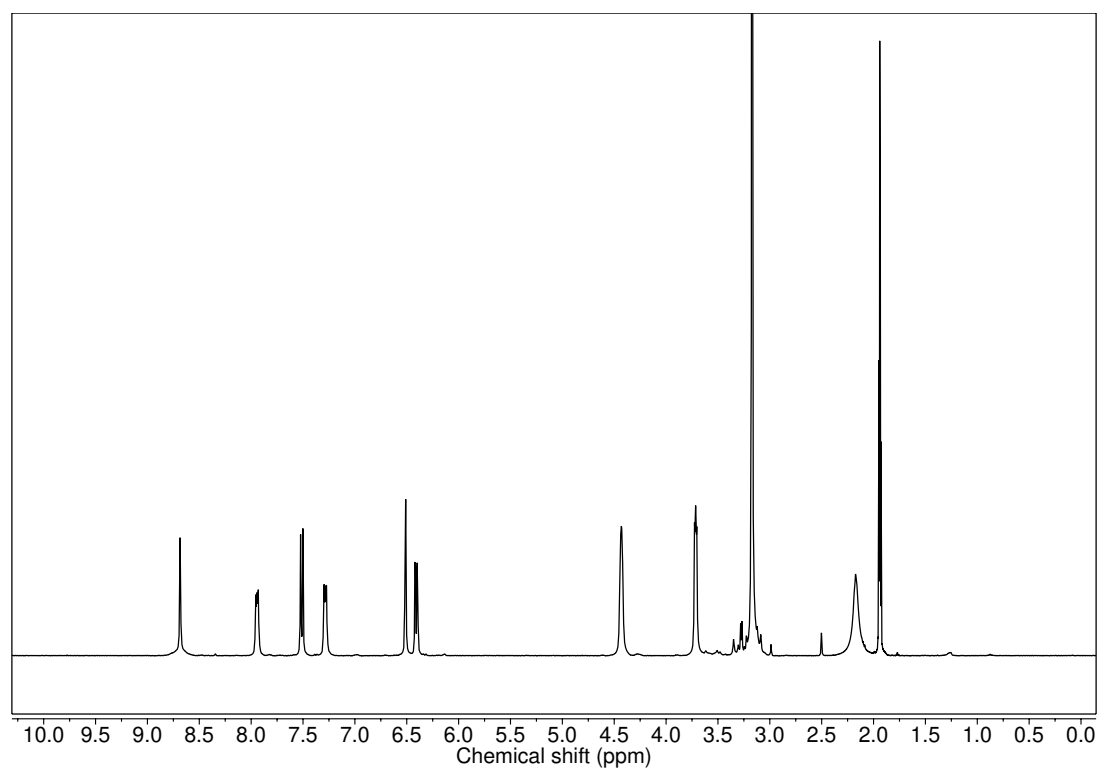


Figure A.56 ^1H NMR spectrum of compound **48** (CD_3CN).

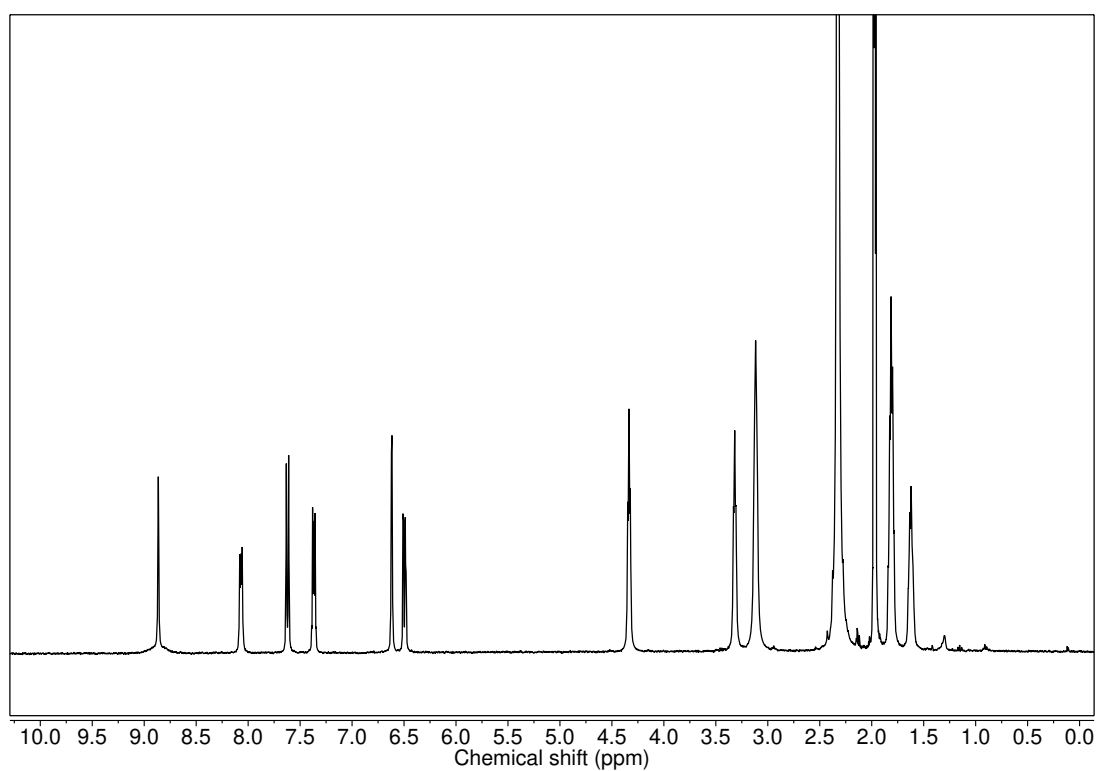


Figure A.57 ^1H NMR spectrum of compound 49 (CD_3CN).

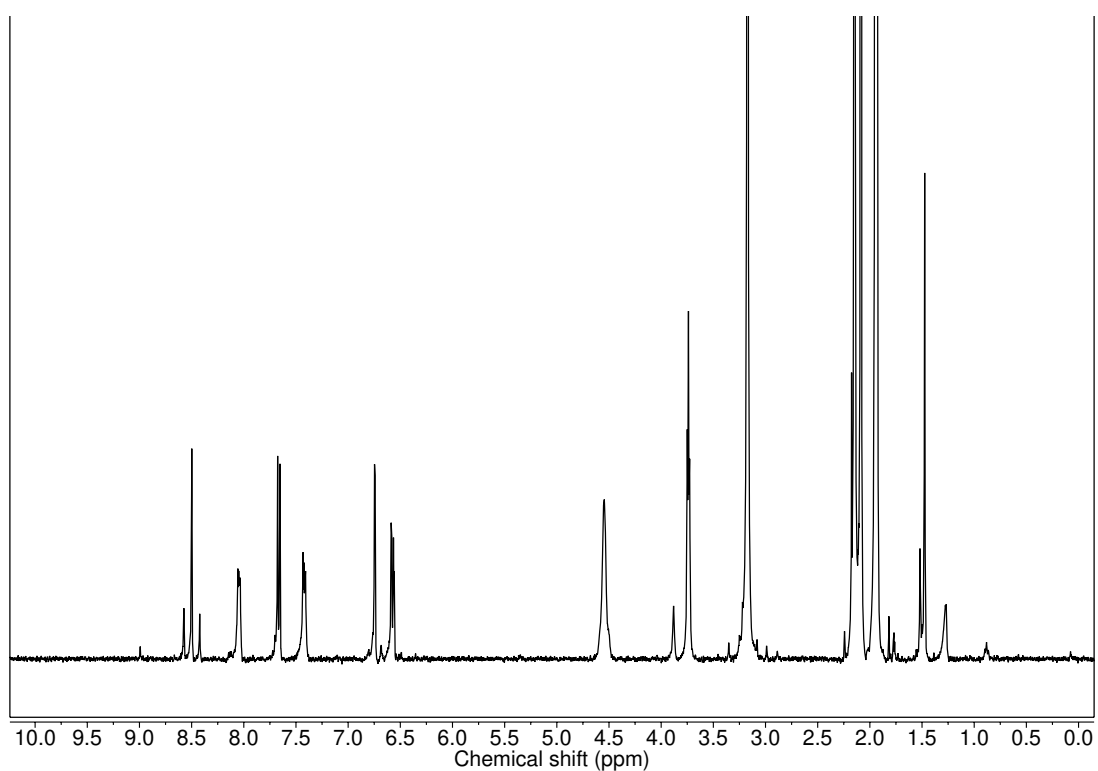


Figure A.58 ^1H NMR spectrum of compound 50 (CD_3CN).

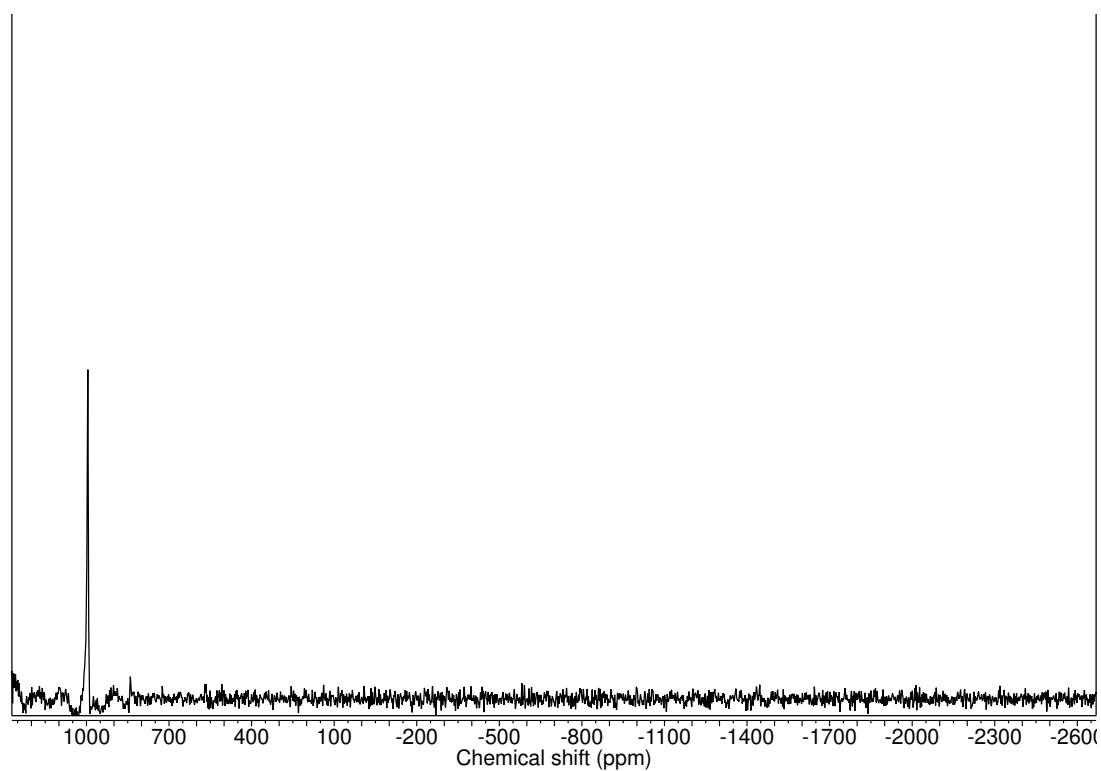


Figure A.59 ^{195}Pt NMR spectrum of compound **50** (CD_3CN).

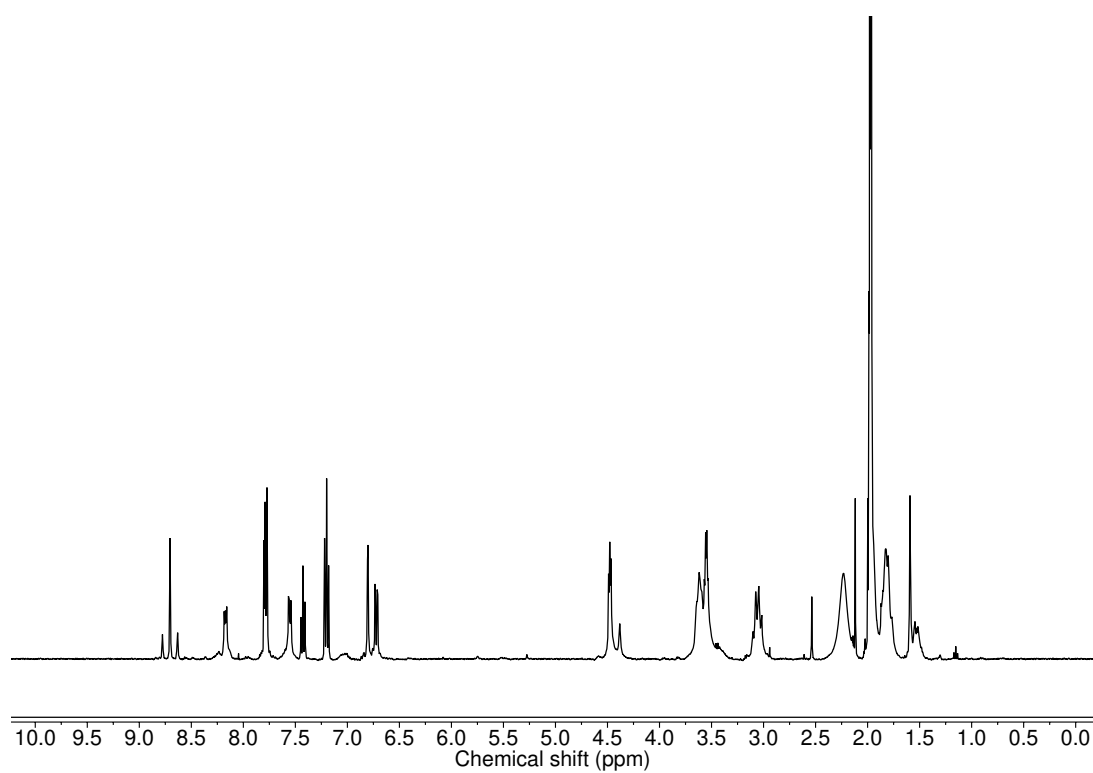


Figure A.60 ^1H NMR spectrum of compound **51** (CD_3CN).

A.2 LCMS data

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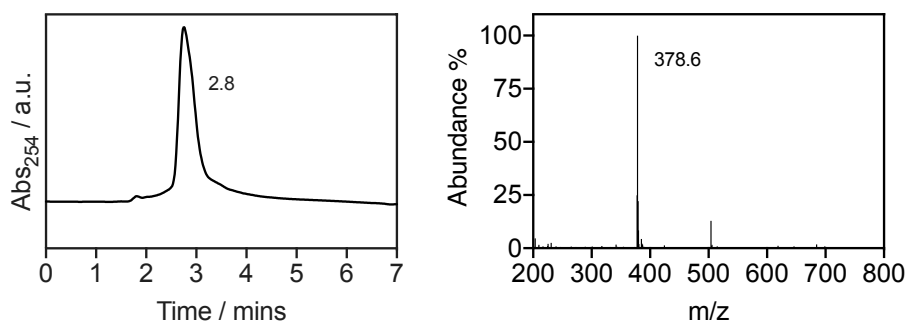


Figure A.61 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 13.

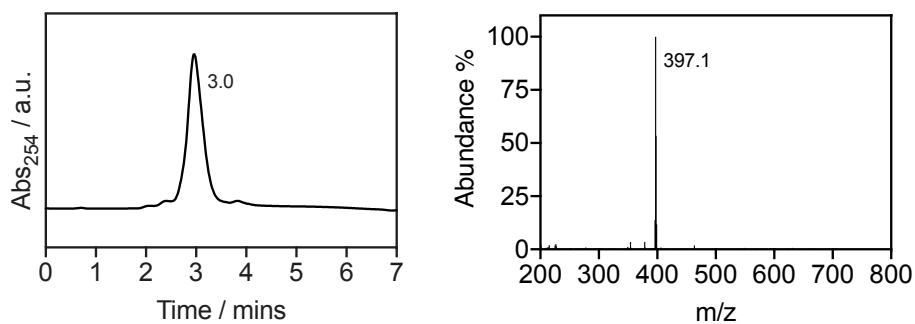


Figure A.62 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 15.

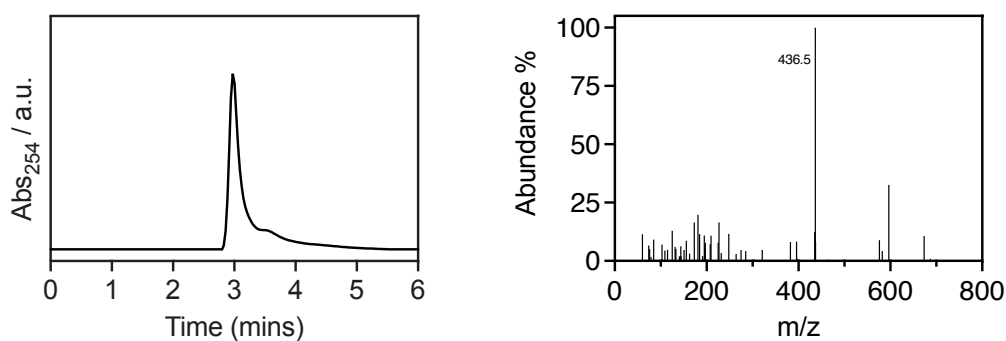


Figure A.63 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 16.

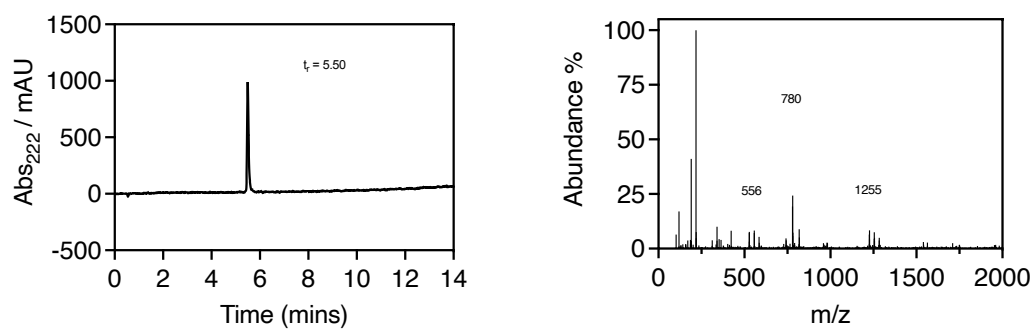


Figure A.64 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 17.

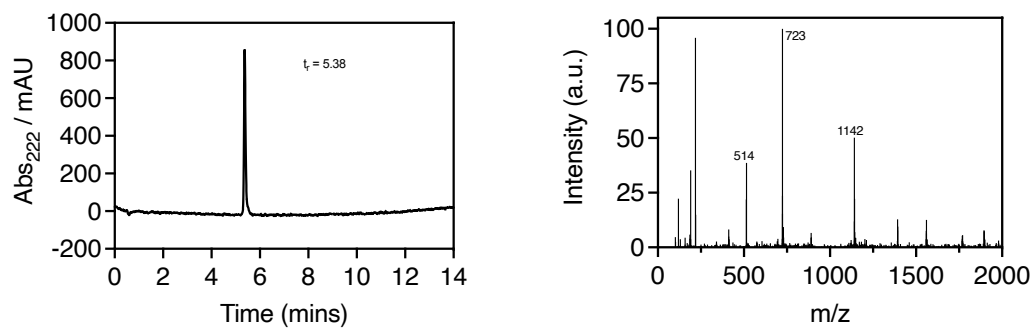


Figure A.65 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 18.

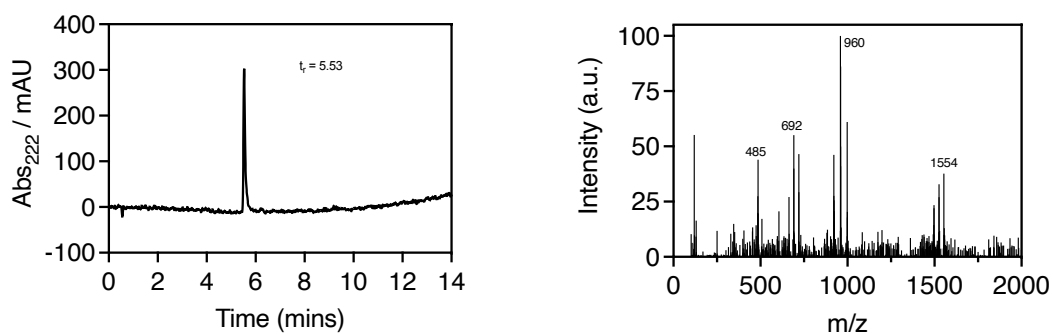


Figure A.66 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 19.

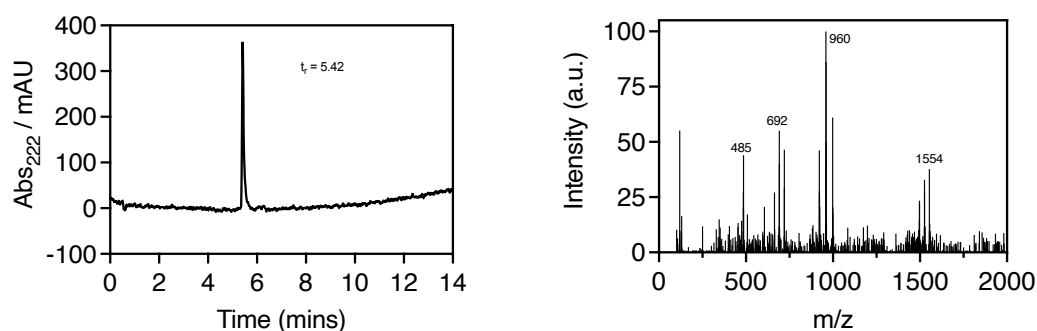


Figure A.67 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 20.

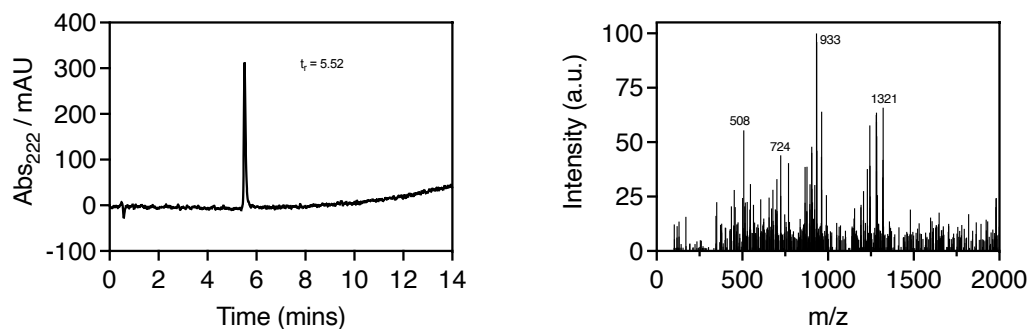


Figure A.68 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 21.

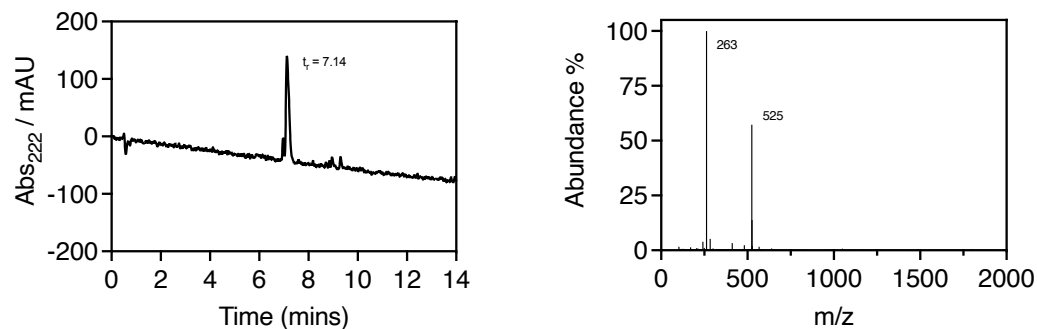


Figure A.69 LCMS data (5–95% MeOH in water with 0.1% TFA) for peptide a.

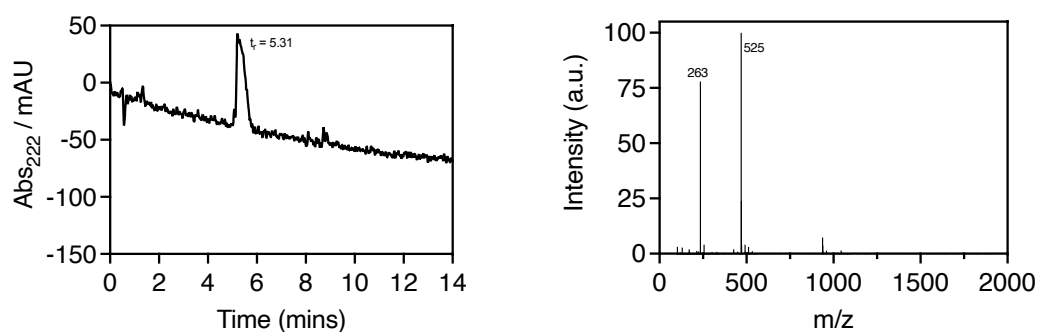


Figure A.70 LCMS data (5–95% MeOH in water with 0.1% TFA) for peptide b.

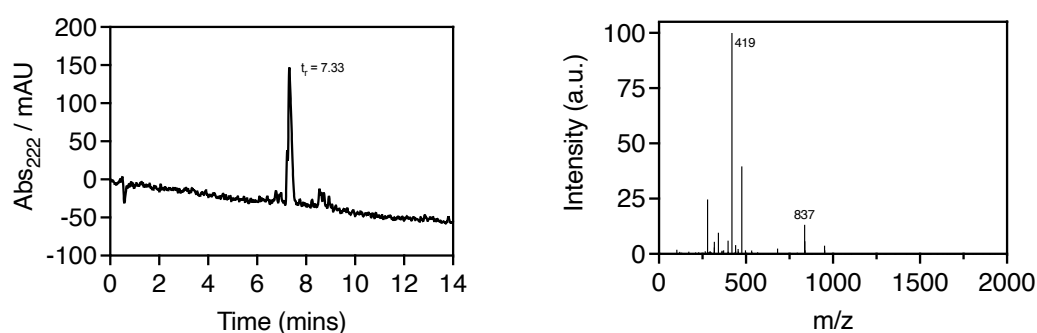


Figure A.71 LCMS data (5–95% MeOH in water with 0.1% TFA) for peptide c.

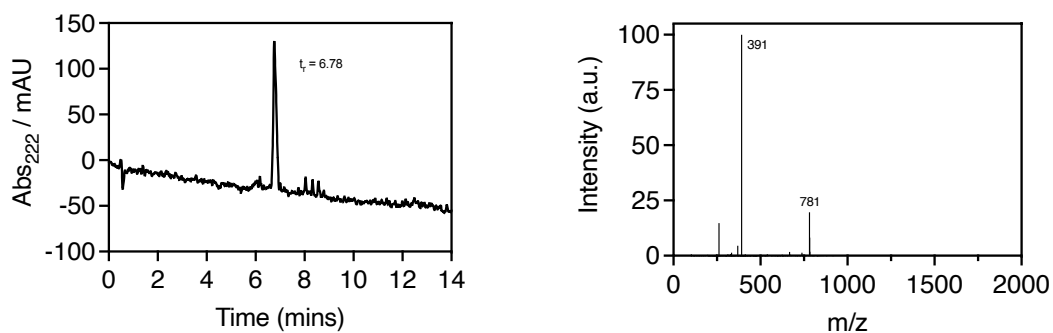


Figure A.72 LCMS data (5–95% MeOH in water with 0.1% TFA) for peptide d.

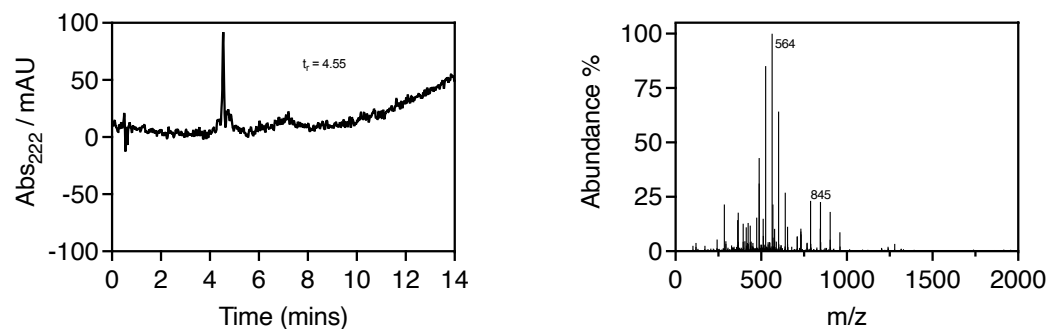


Figure A.73 LCMS data (5–95% MeOH in water with 0.1% TFA) for peptide e.

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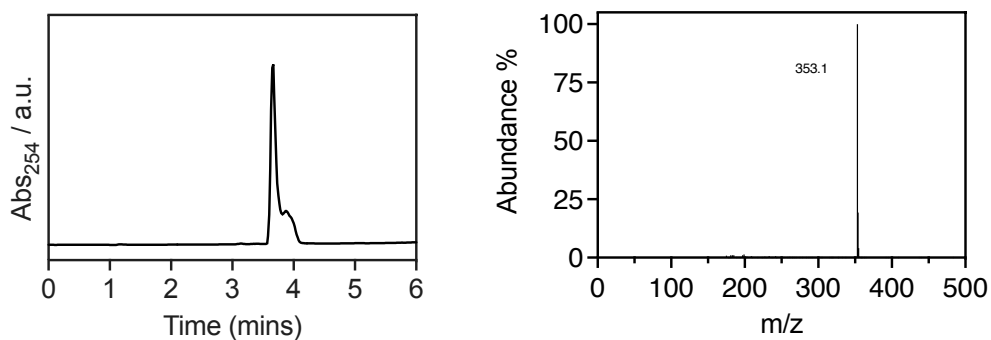


Figure A.74 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 31.

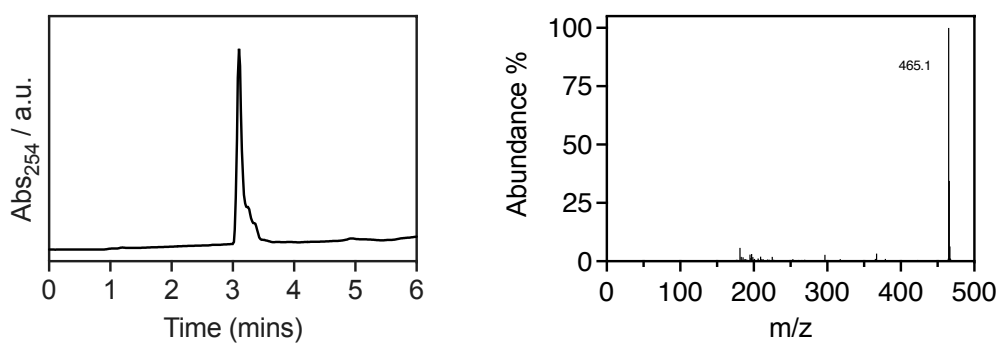


Figure A.75 LCMS data (5–95% MeOH in water with 0.1% TFA) for compound 34.

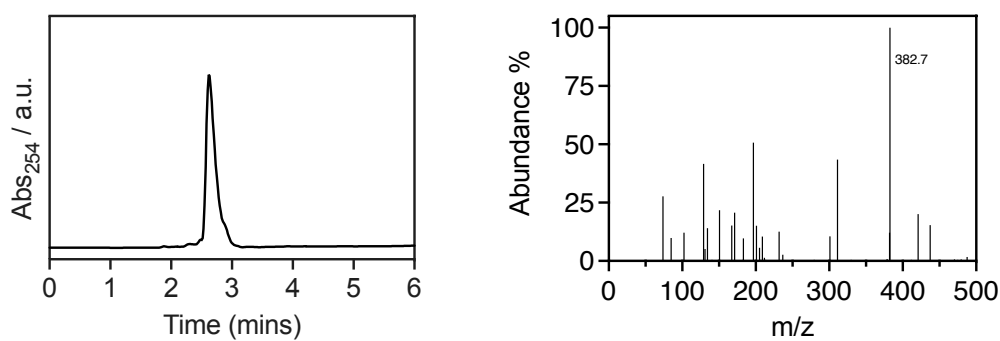


Figure A.76 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 35.

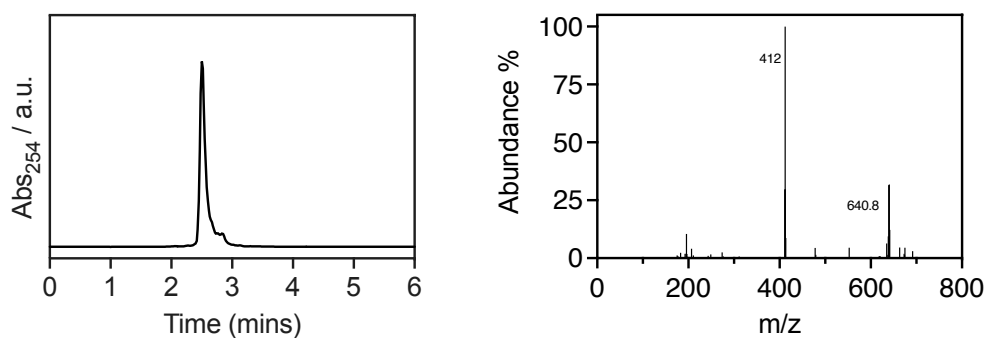


Figure A.77 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 36.

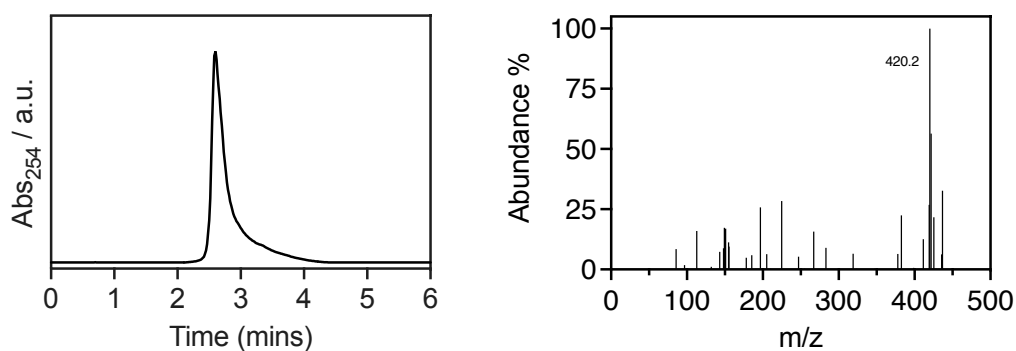


Figure A.78 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 37.

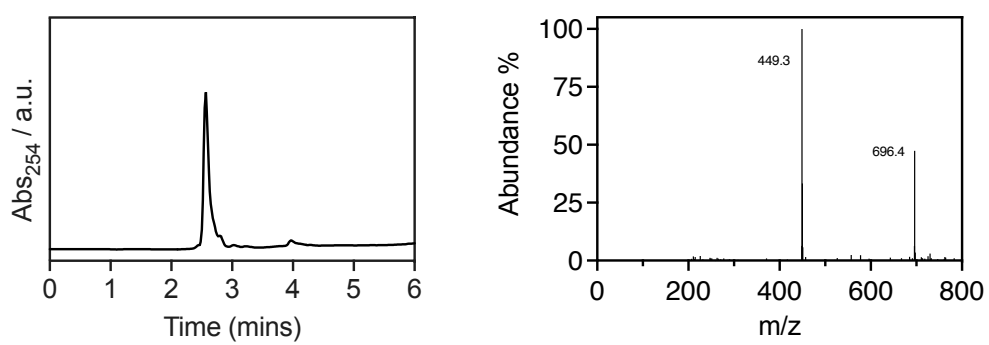


Figure A.79 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound **38**.

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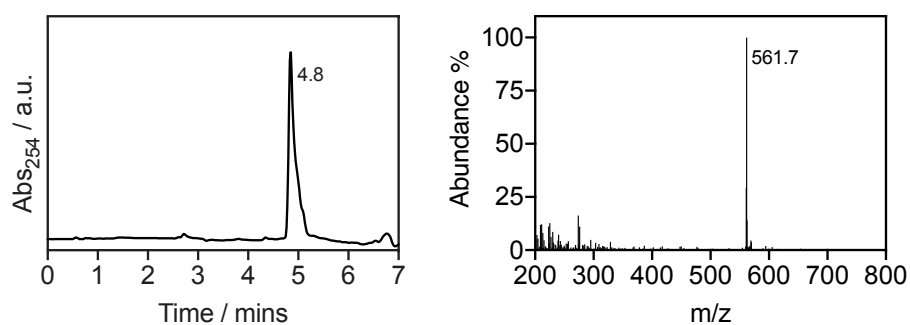


Figure A.80 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 46^{enz}.

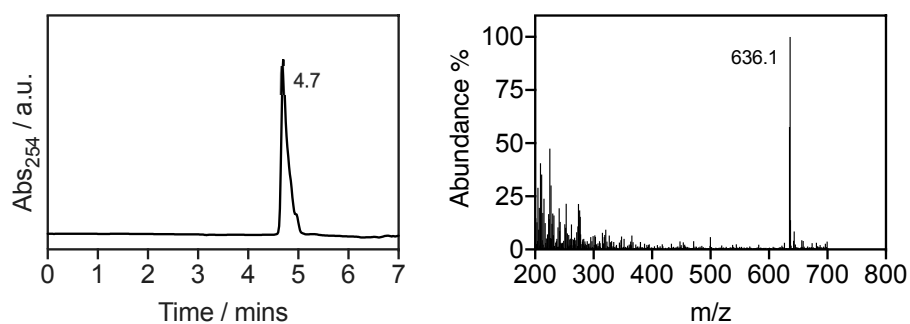


Figure A.81 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound 46^{photo}.

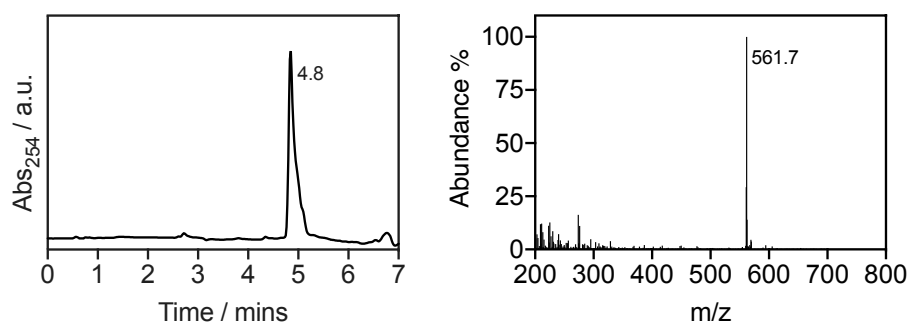


Figure A.82 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound **46^{ctrl}**.

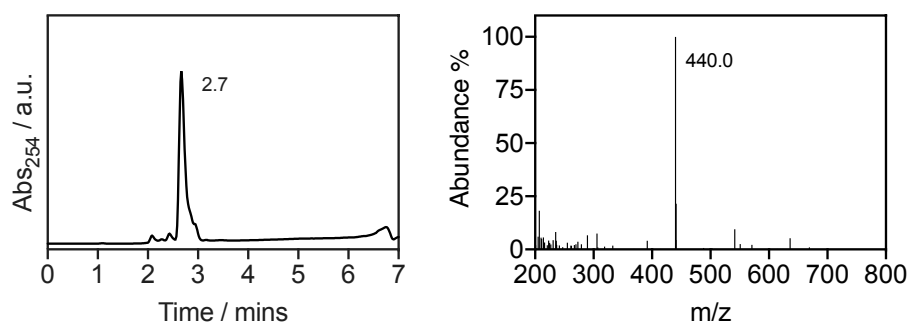


Figure A.83 LCMS data (5–95% MeOH in water with 0.1% formic acid) for compound **47**

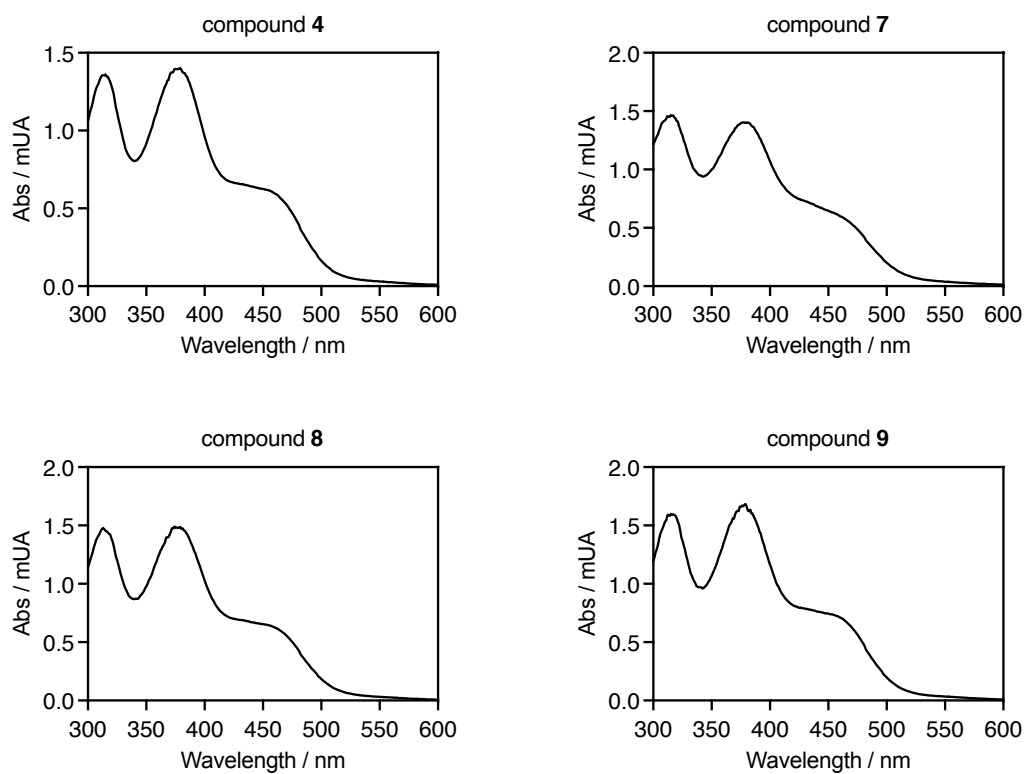
A.3 Absorbance spectra

Figure A.84 Absorbance spectra of Ni^{II}-salphen compounds.

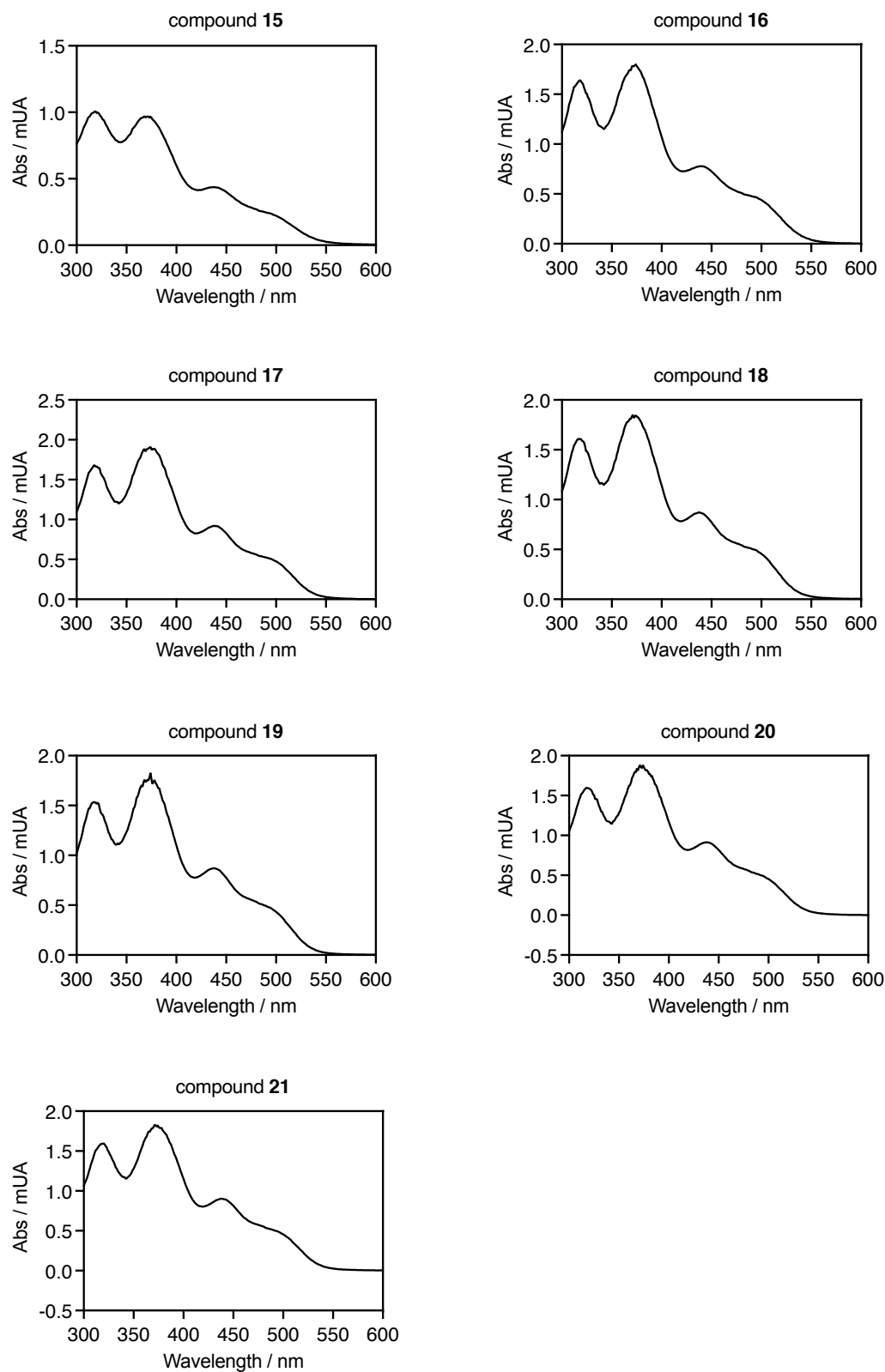


Figure A.85 Absorbance spectra of Pt^{II}-salphen compounds.

A.4 CD melting experiments

A.4.1 Chapter 2

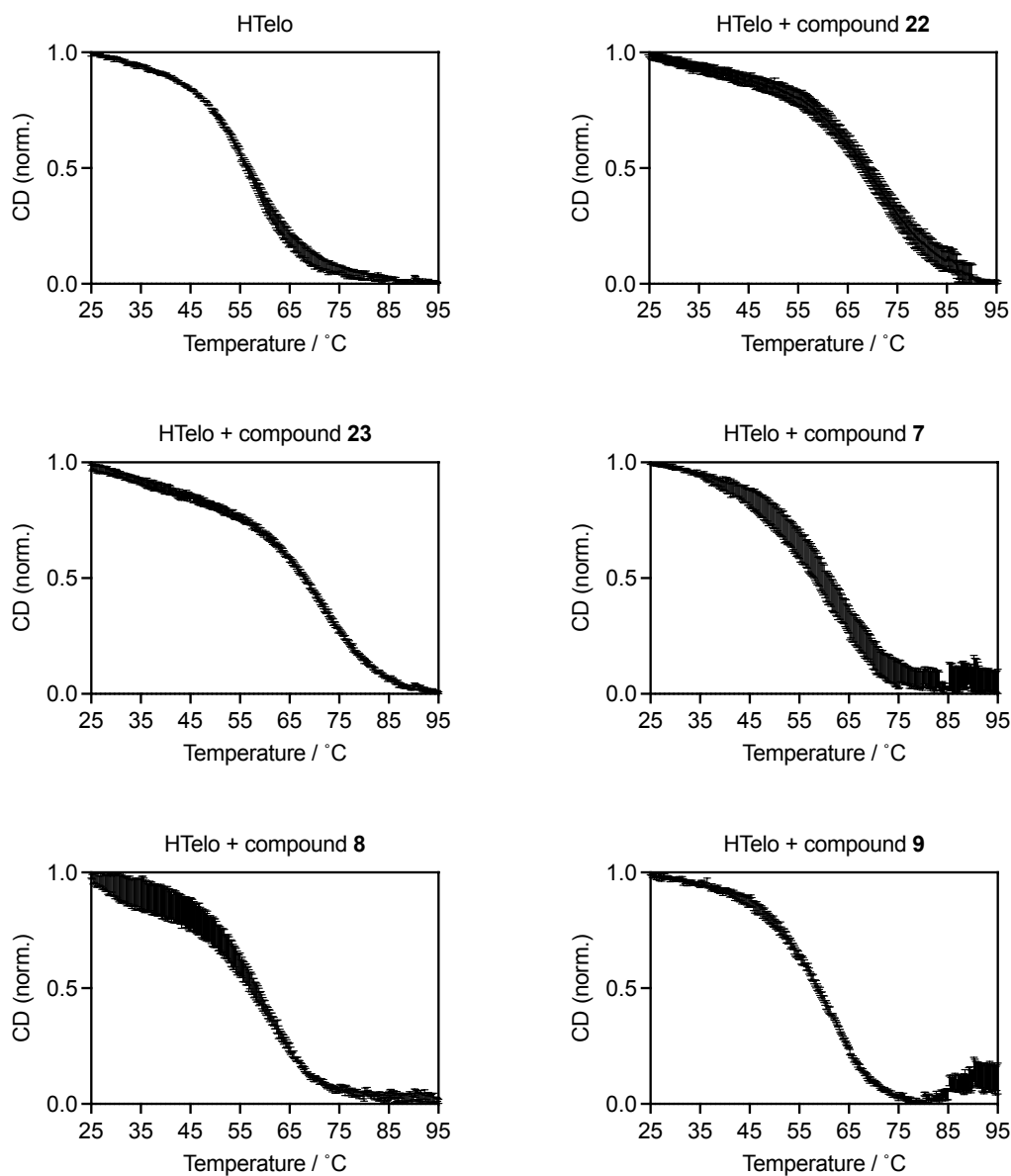


Figure A.86 CD melting experiments of Ni^{II}-salphens with G1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

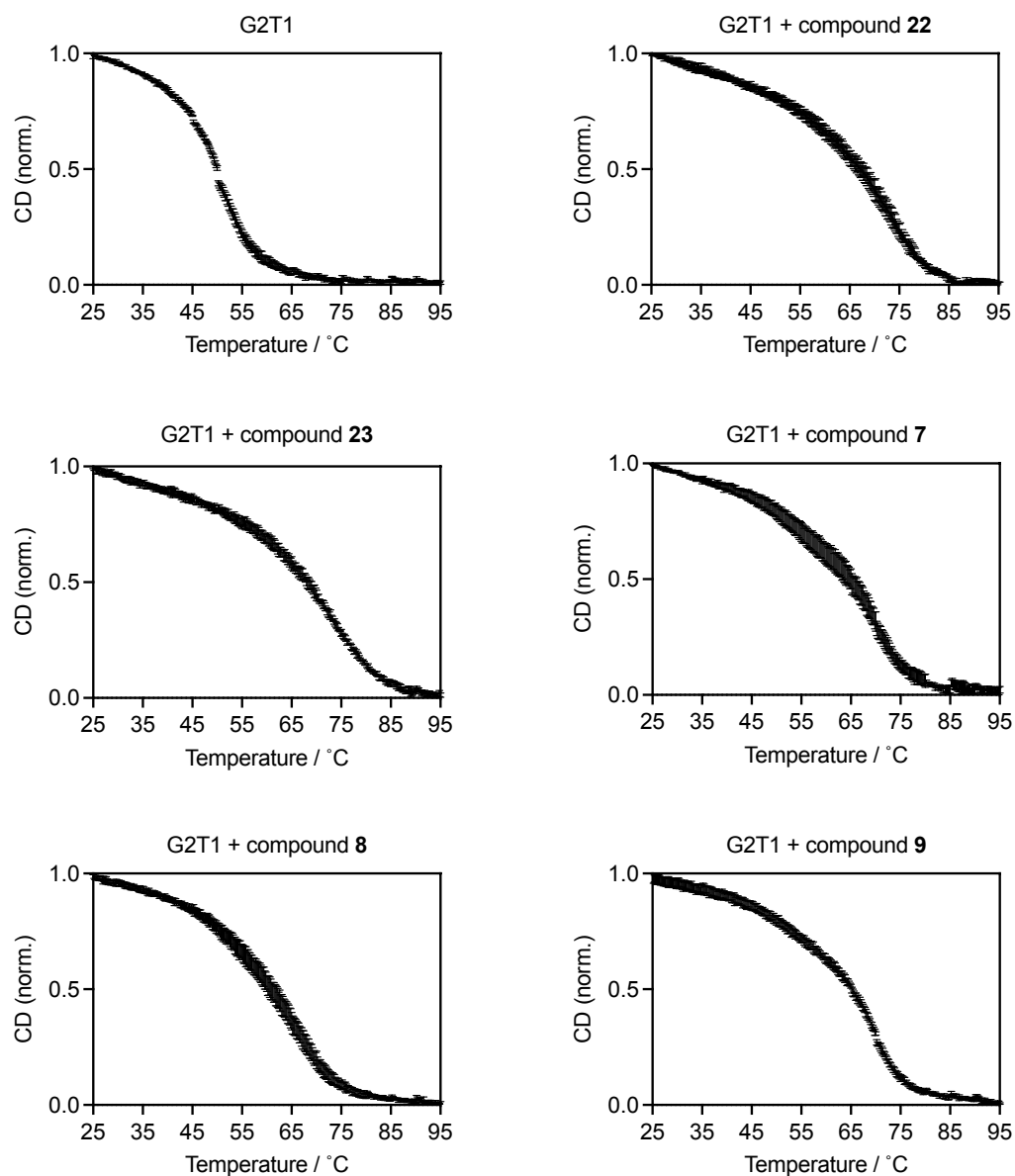


Figure A.87 CD melting experiments of Ni^{II}-salphens with G2T1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

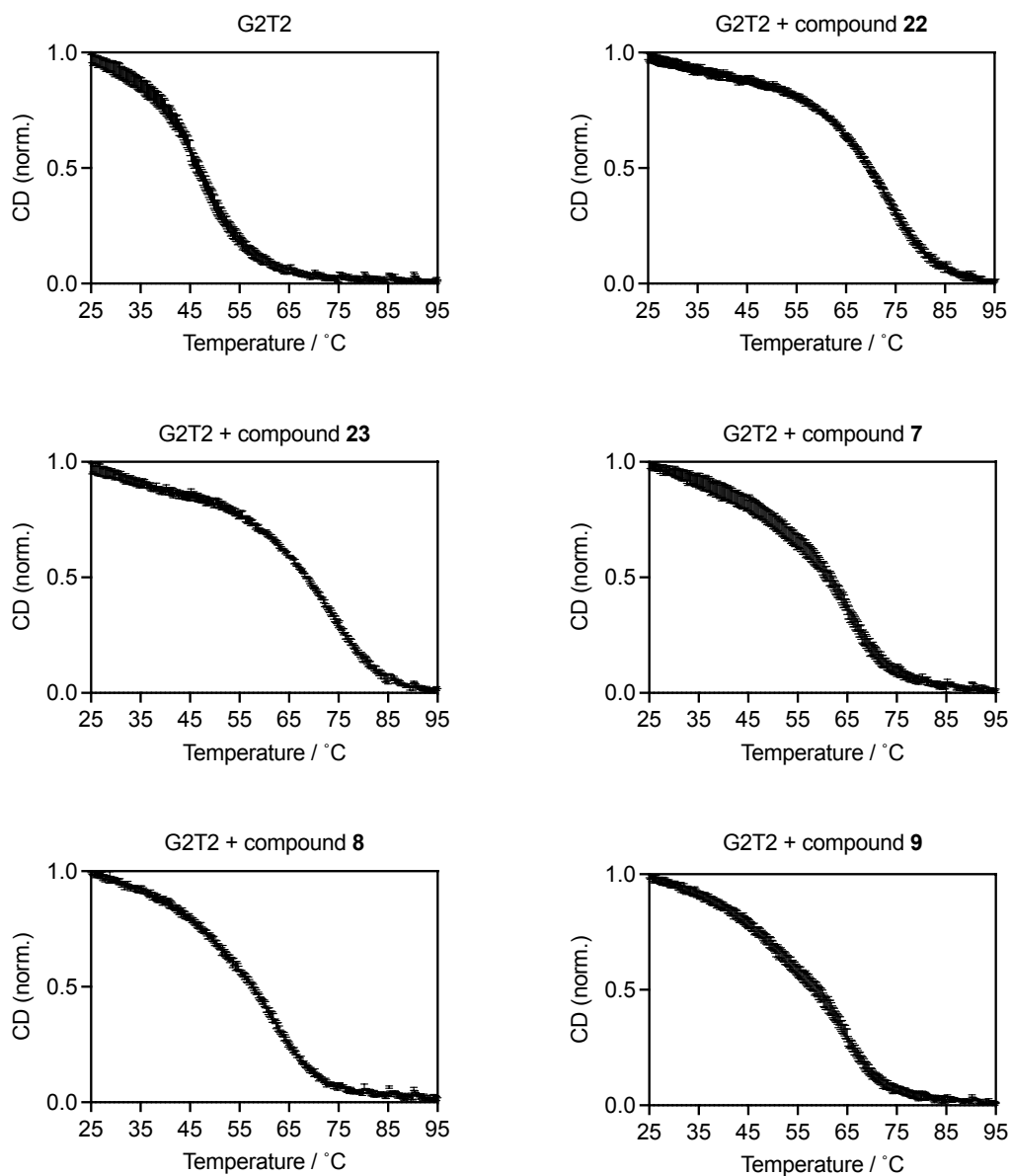


Figure A.88 CD melting experiments of Ni^{II}-salphens with G2T2 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

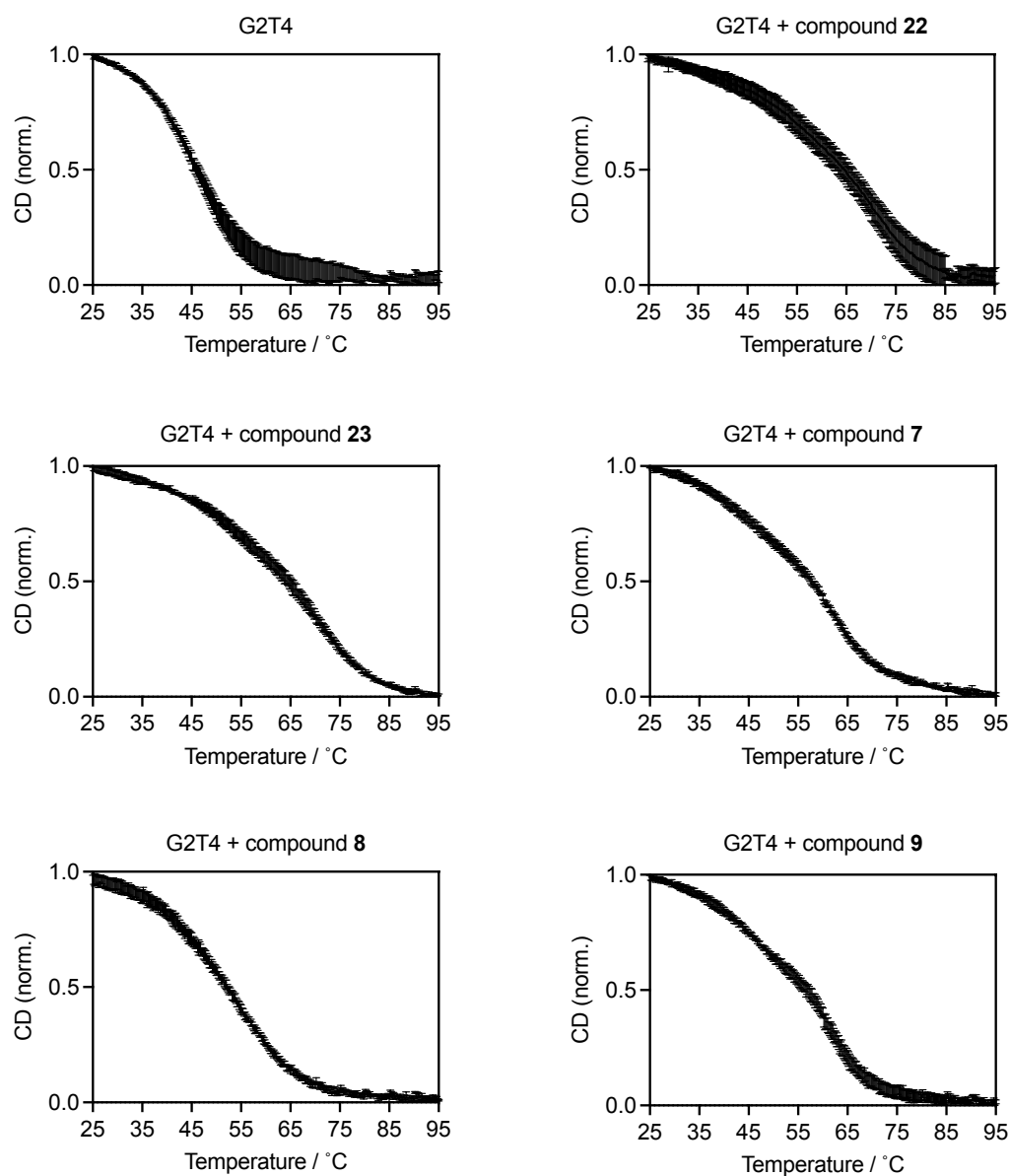


Figure A.89 CD melting experiments of Ni^{II}-salphens with G2T4 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

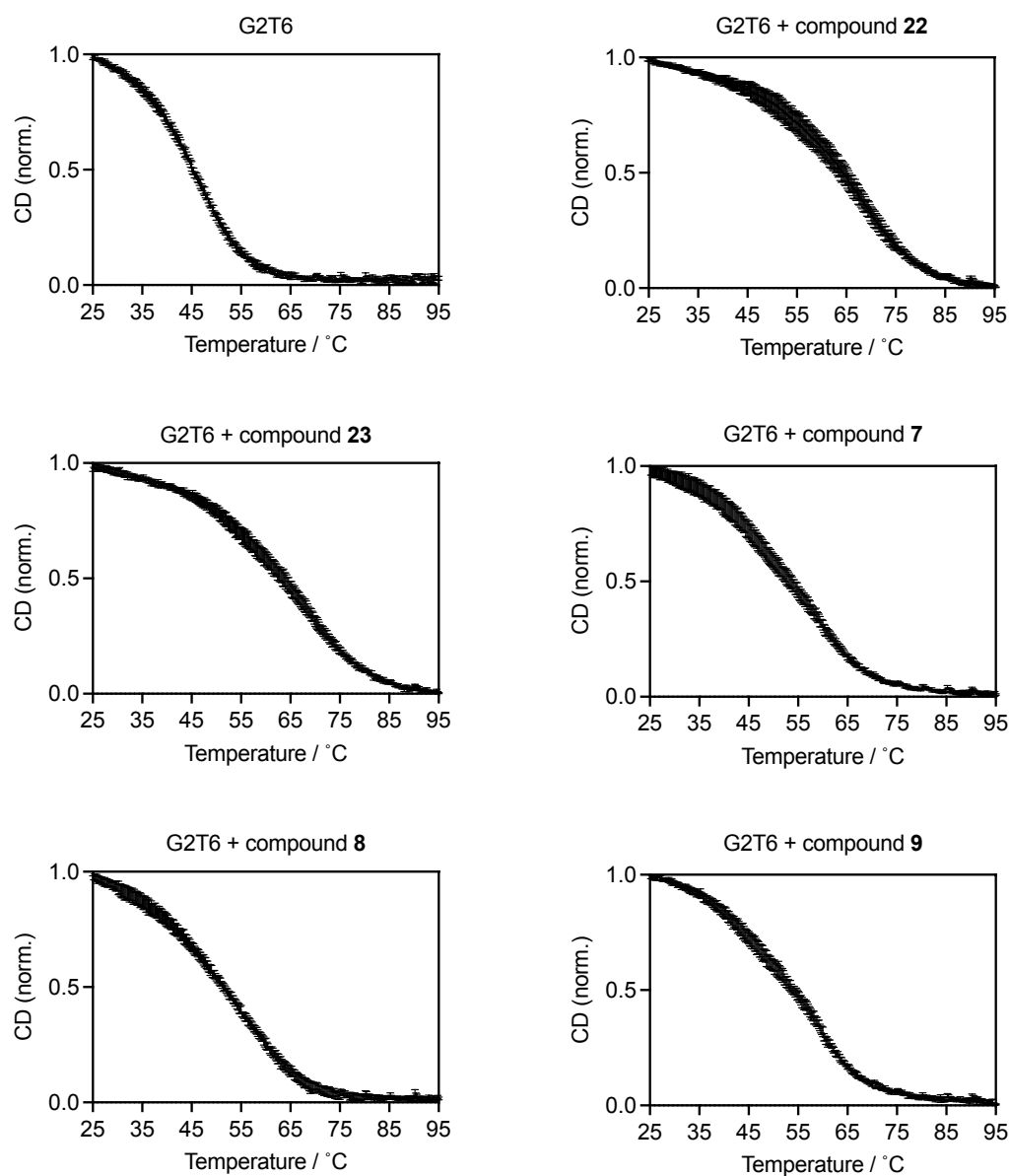


Figure A.90 CD melting experiments of Ni^{II}-salphens with G2T6 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

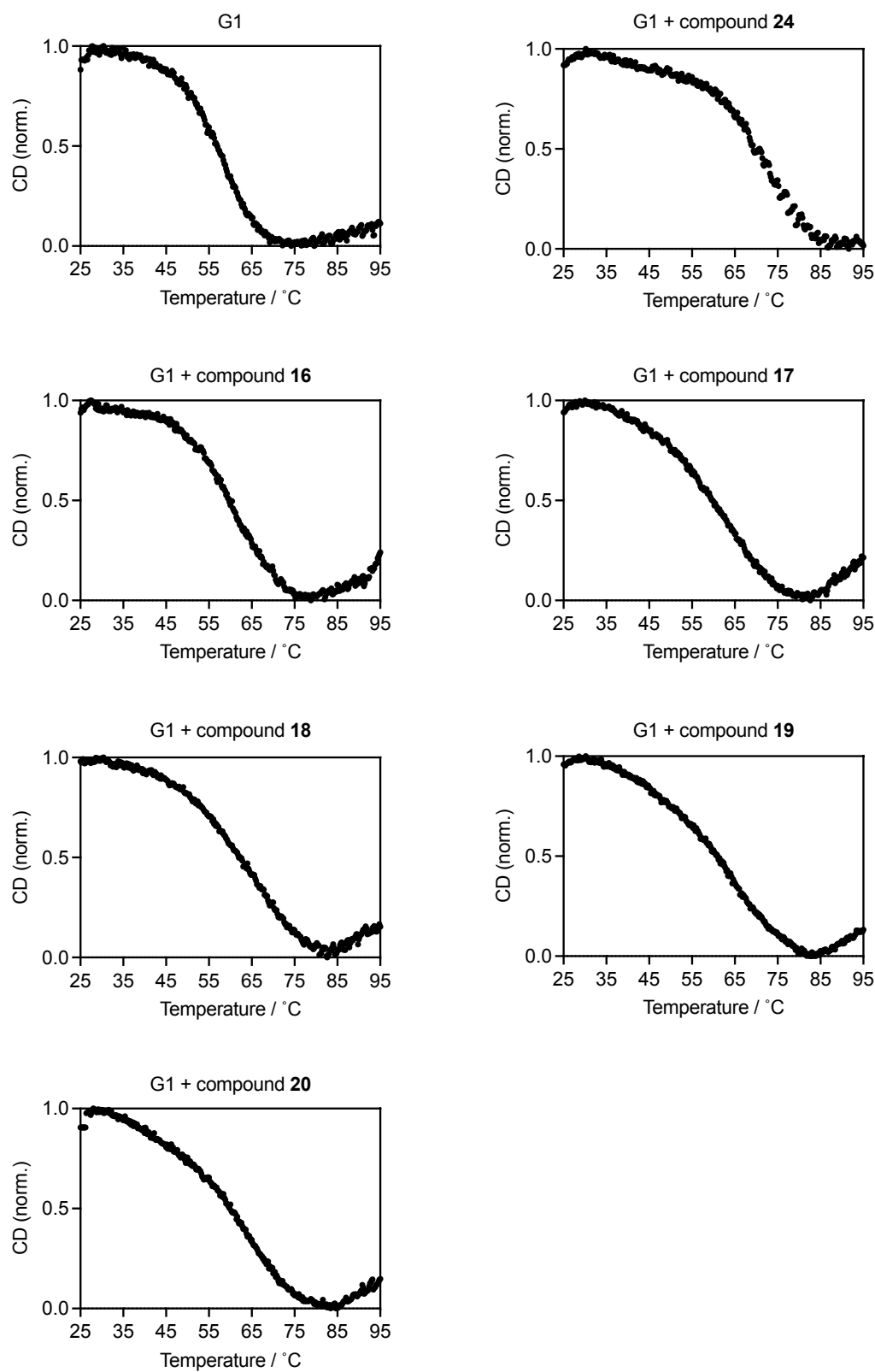


Figure A.91 CD melting experiments of Pt^{II}-salphens with G1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

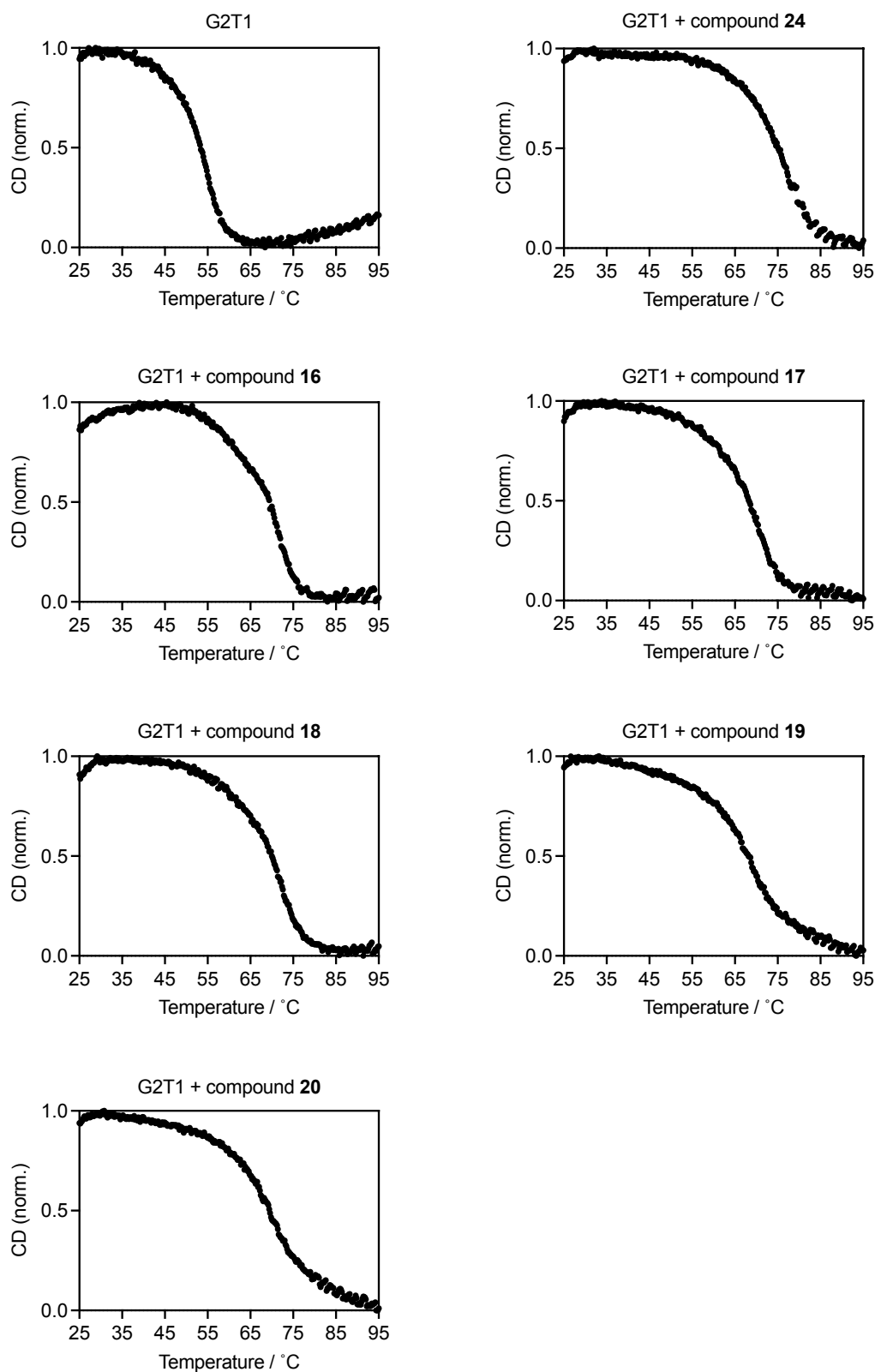


Figure A.92 CD melting experiments of Pt^{II}-salphen with G2T1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

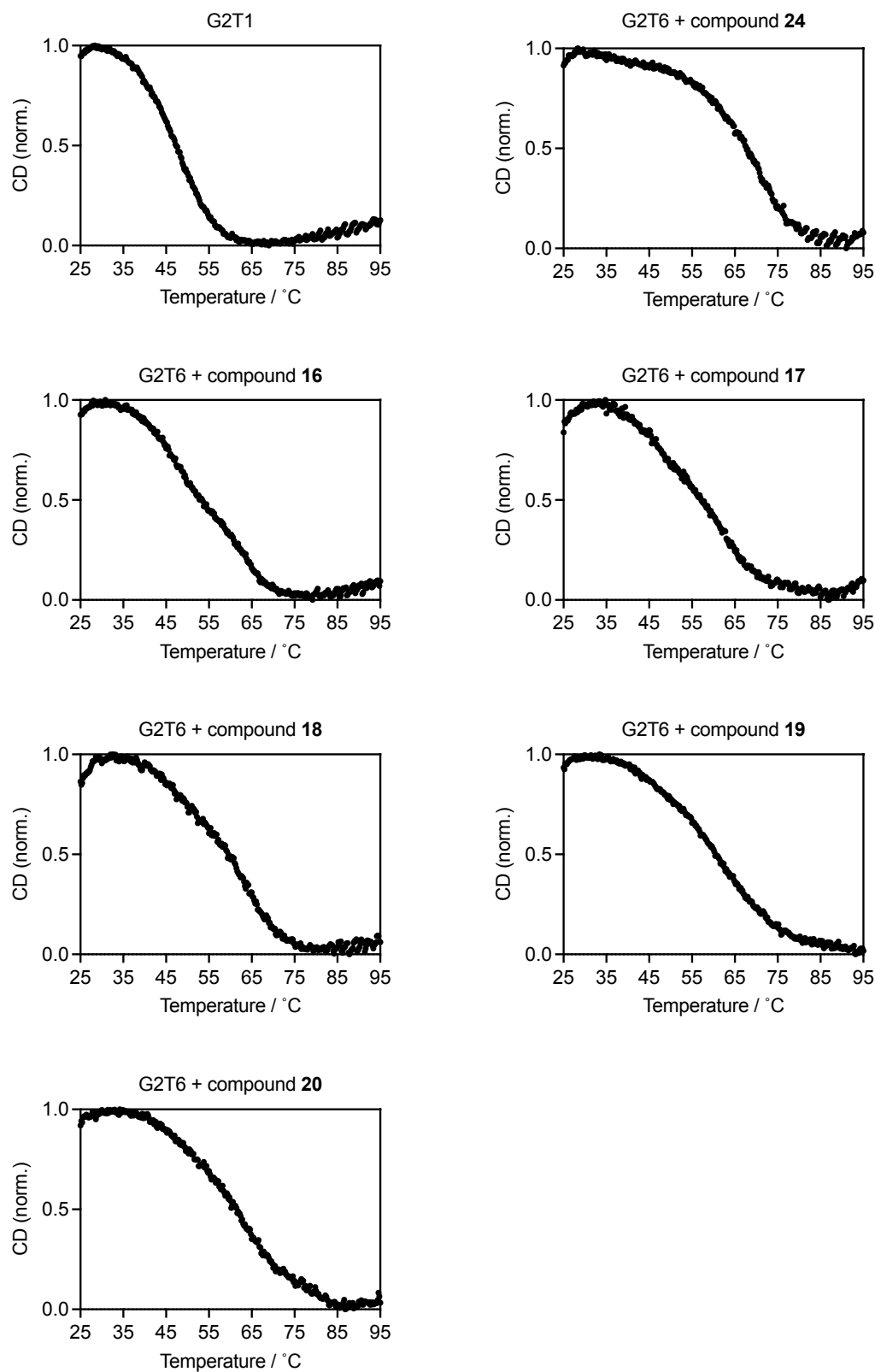


Figure A.93 CD melting experiments of Pt^{II}-salphen with G2T6 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

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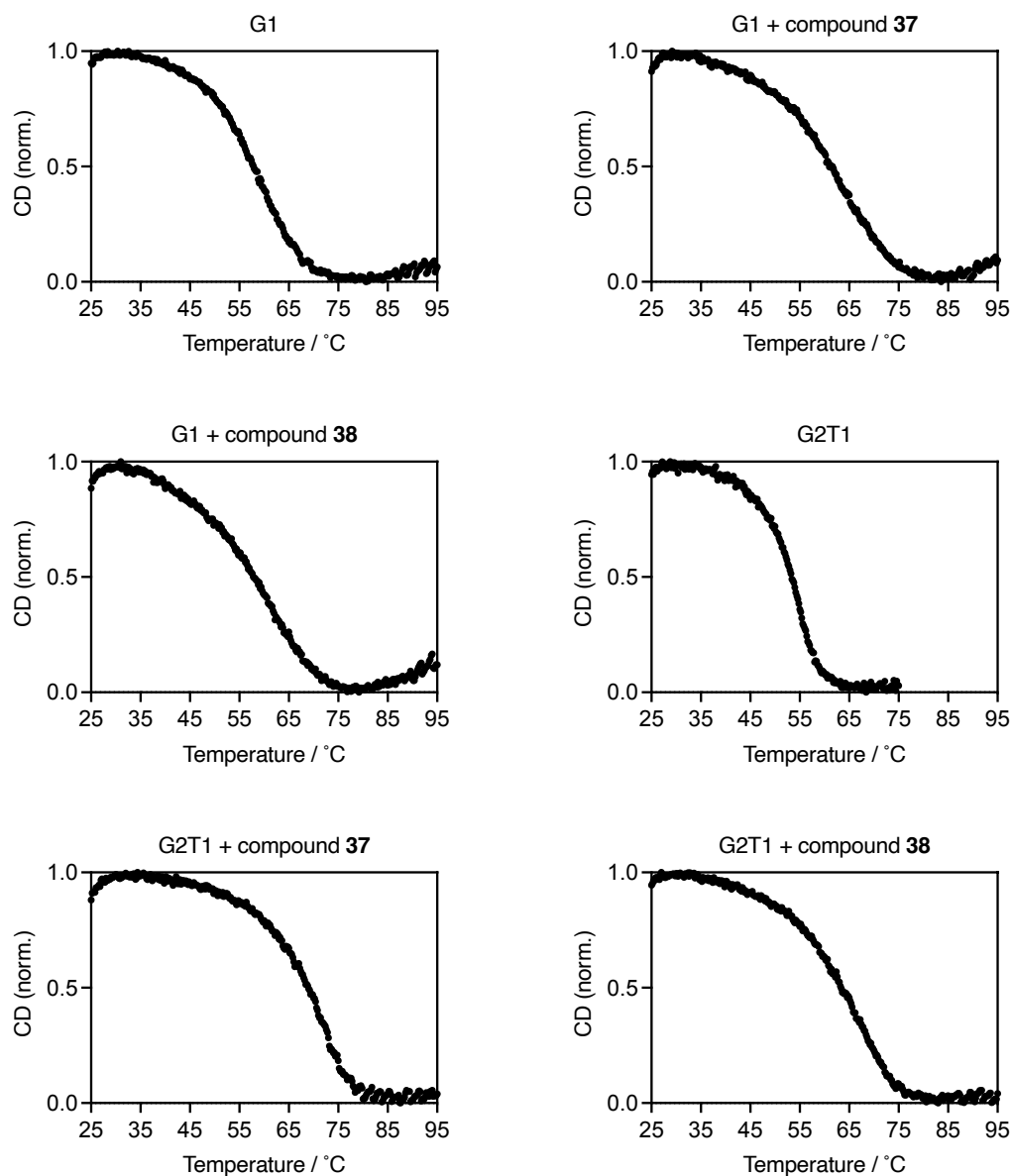


Figure A.94 CD melting experiments of compound **37** and **38** with G1 and G2T1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

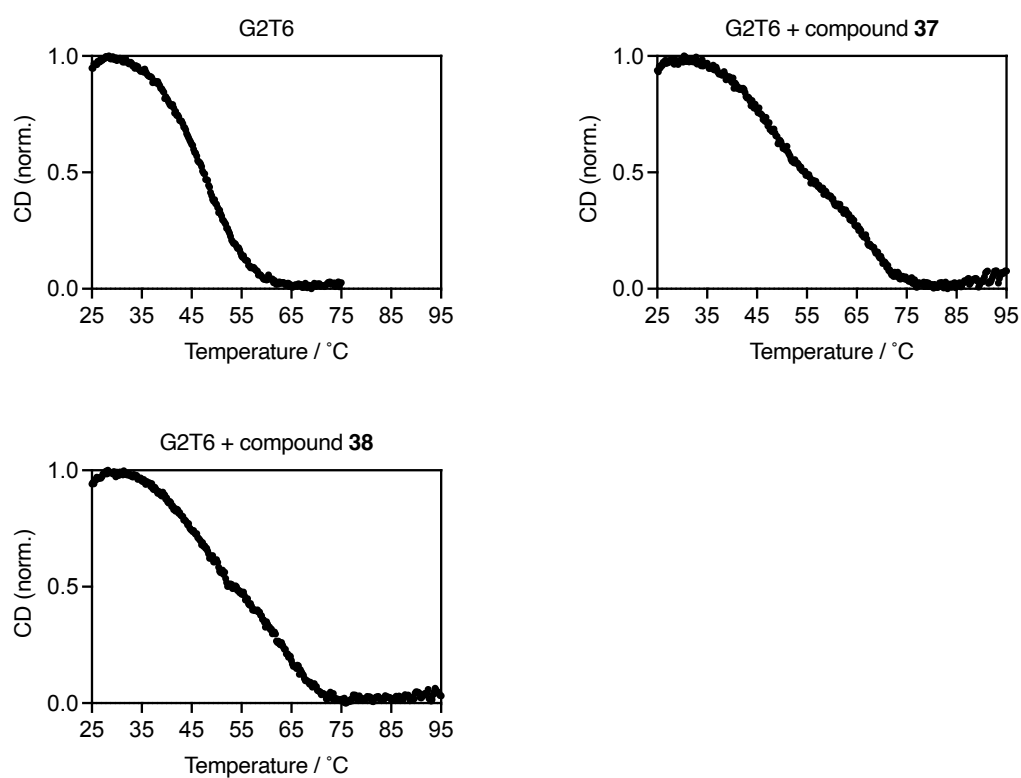


Figure A.95 CD melting experiments of compound **37** and **38** with G2T6 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

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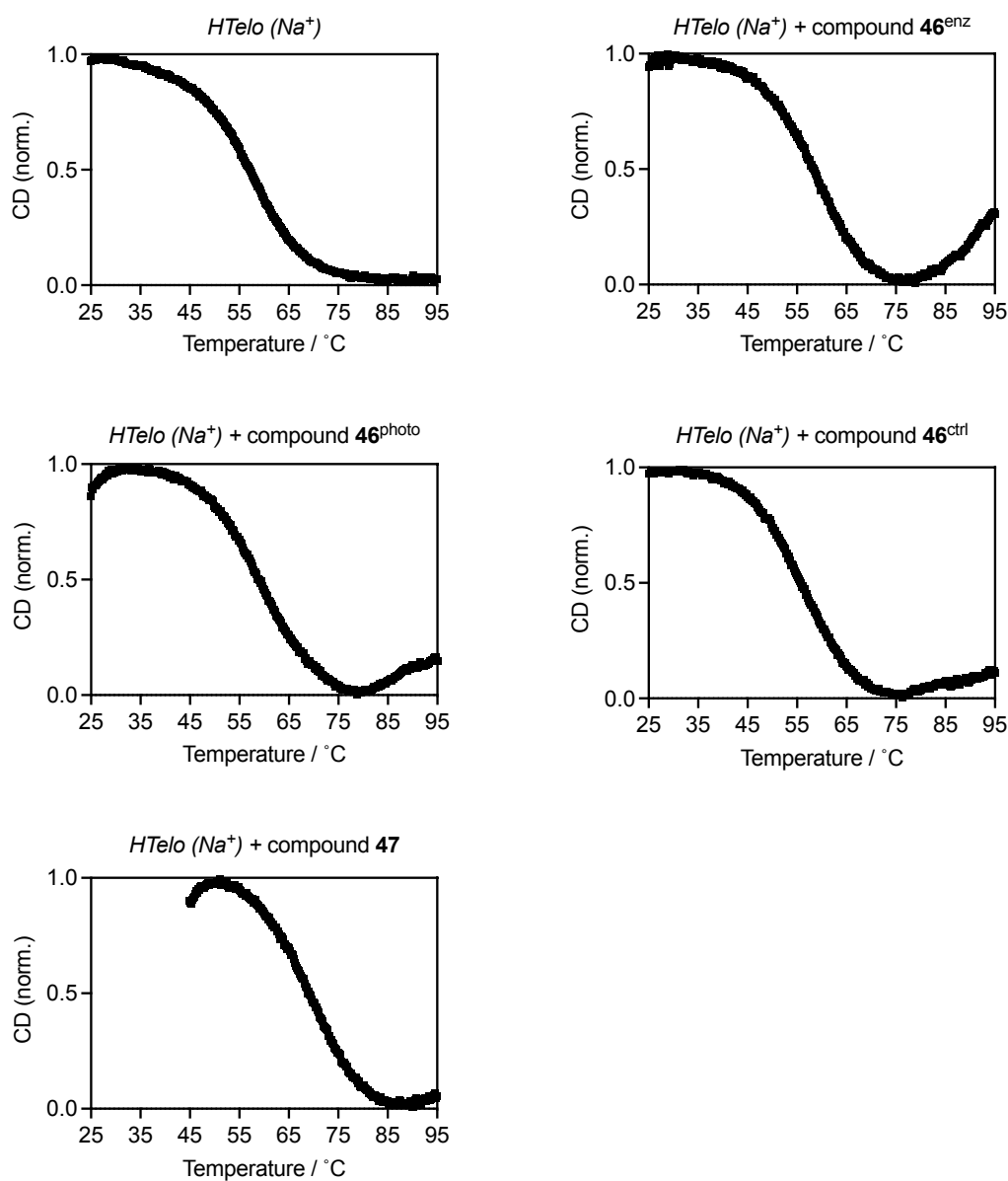


Figure A.96 CD melting experiments of **46^{enz}**, **46^{photo}**, **46^{ctrl}** and compound **47** with G1 DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [G4 unit] = 5 μ M, [salphen unit] = 10 μ M.

A.5 Fluorescence titrations

A.5.1 Chapter 2

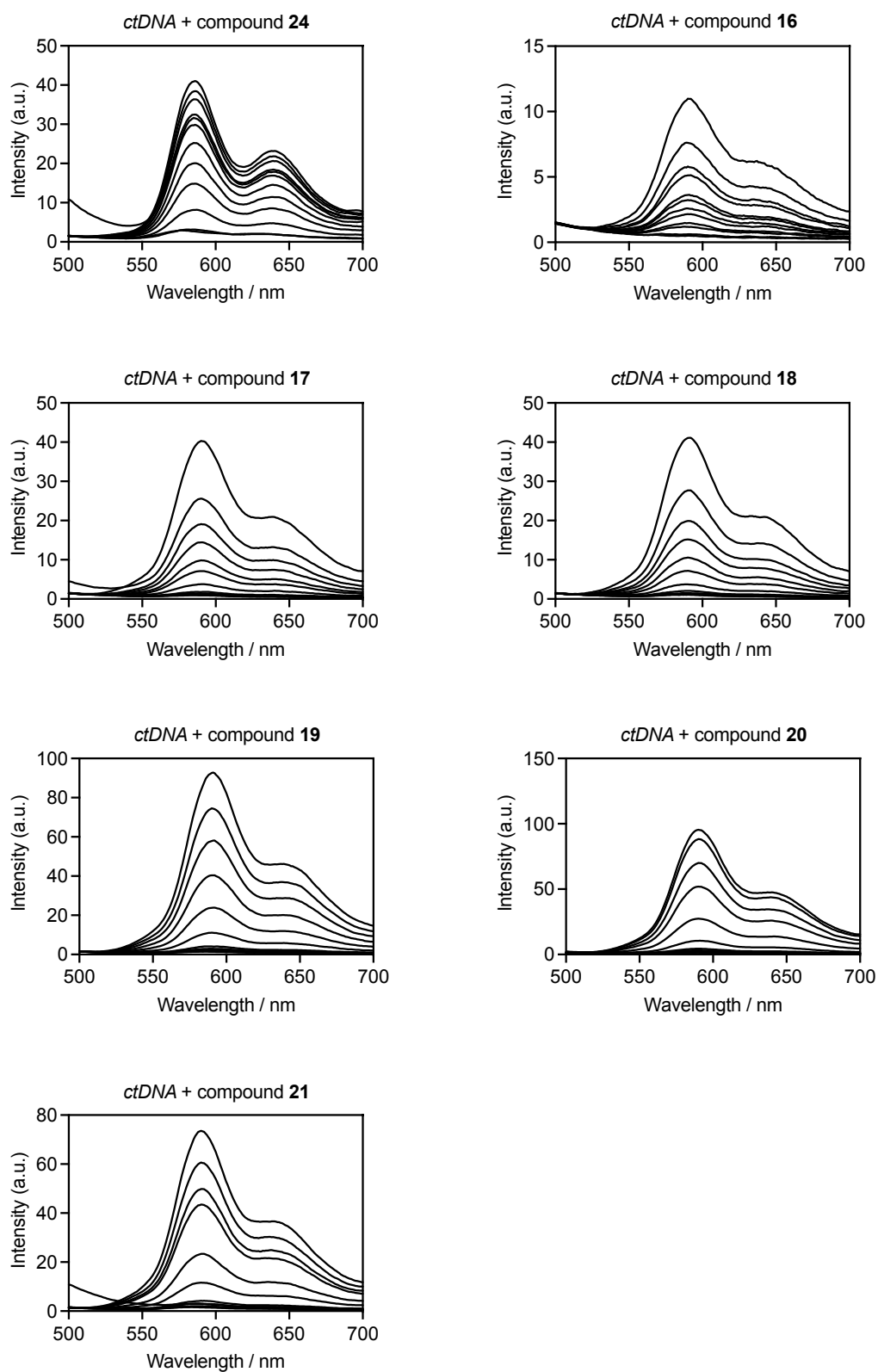


Figure A.97 Emission titrations of Pt^{II}-salphens with CT-DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0-100 μ M base pairs.

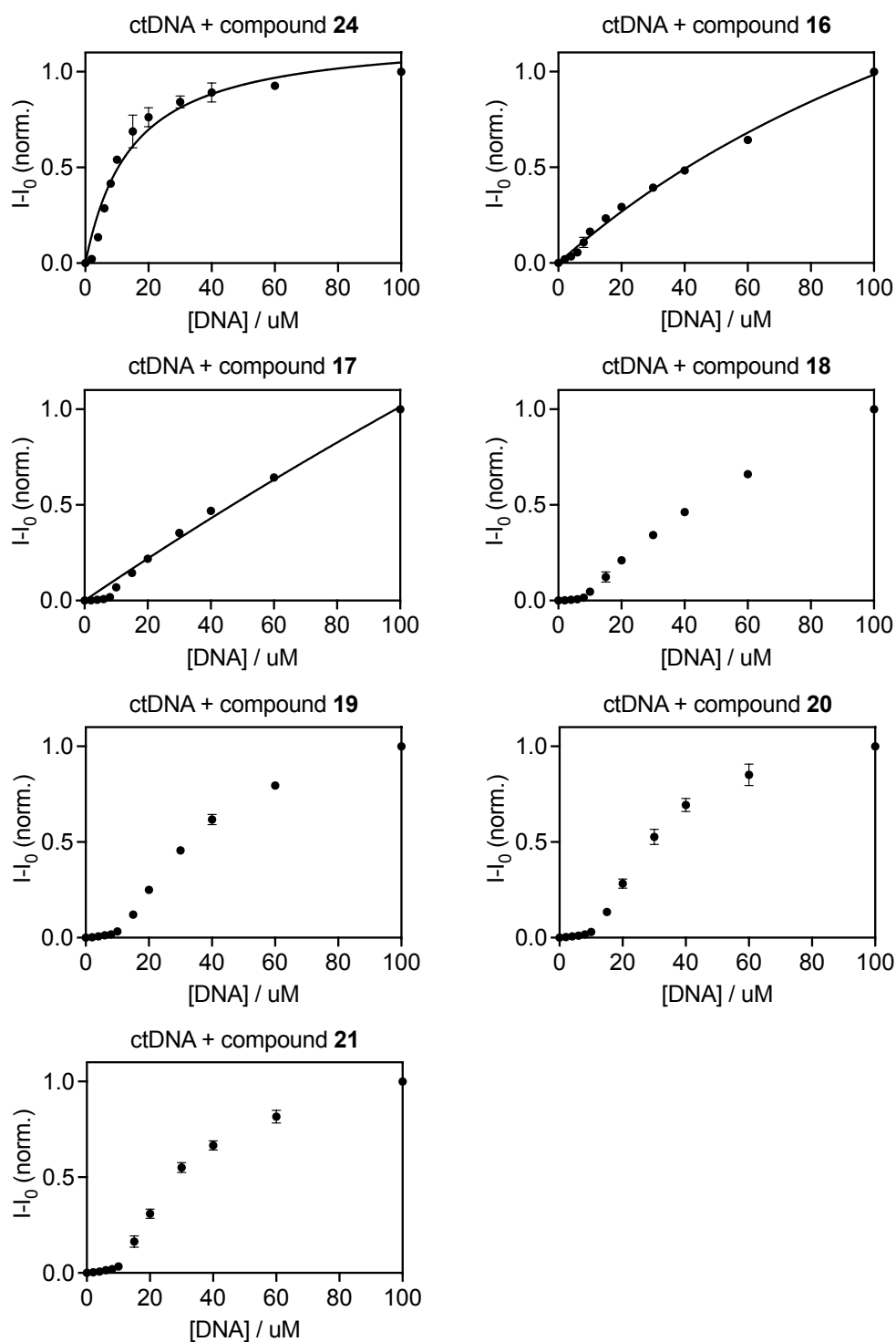


Figure A.98 Fitted data for emission titrations of Pt^{II}-salphens with CT-DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-100 μM base pairs.

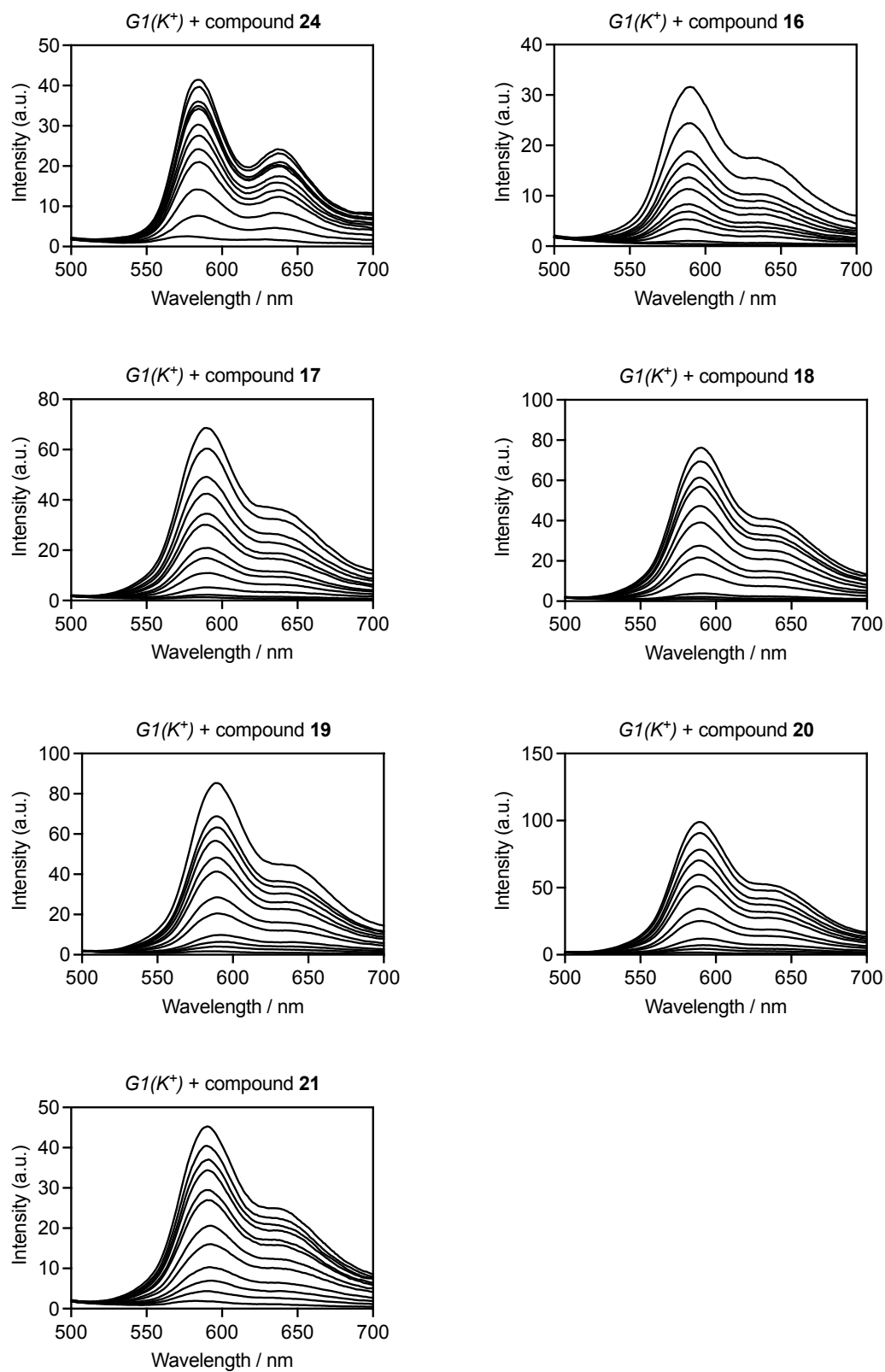


Figure A.99 Emission titrations of Pt^{II}-salphens with G1(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0–10 μ M.

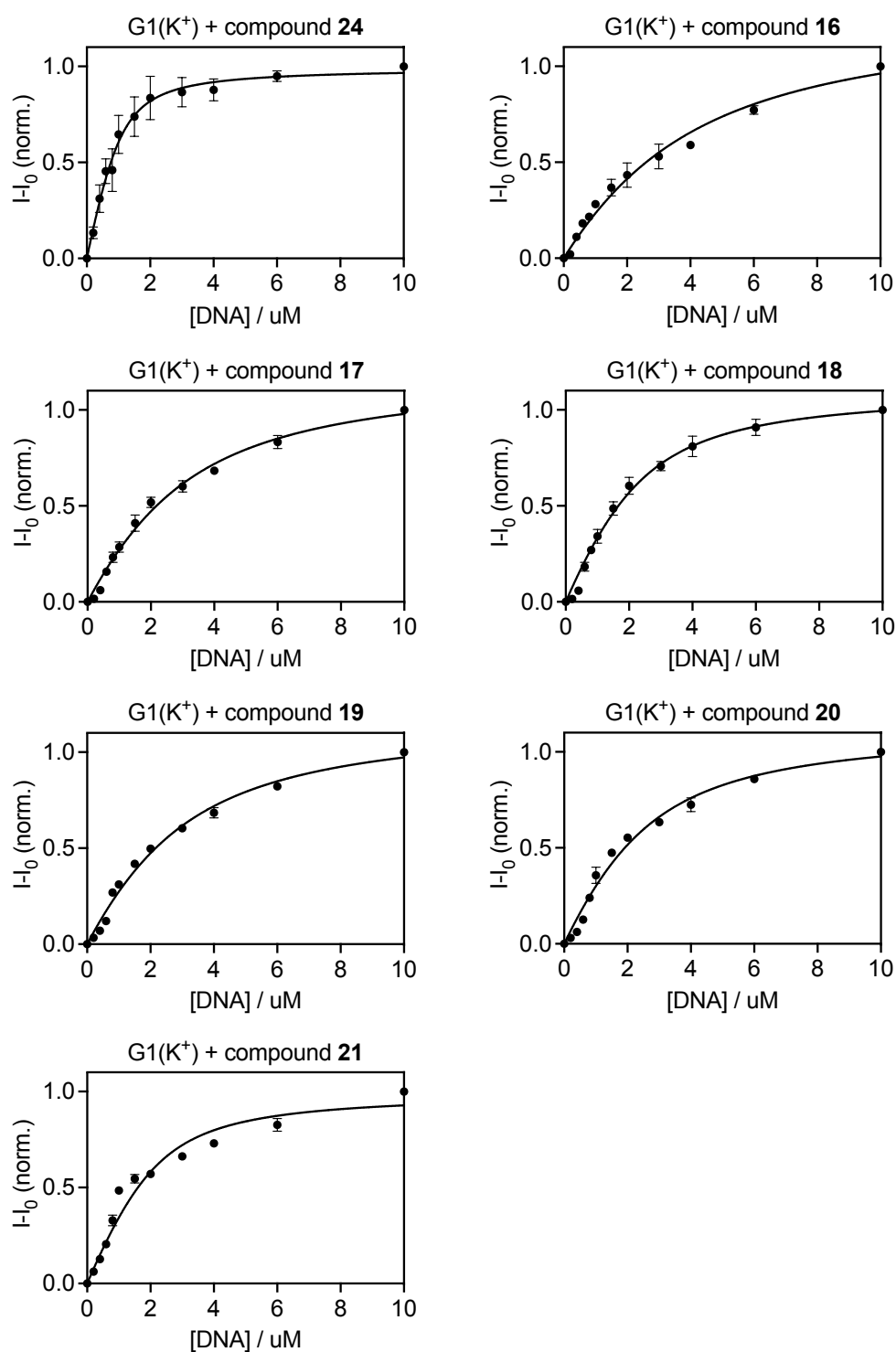


Figure A.100 Fitted data for emission titrations of Pt^{II}-salphens with G1(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

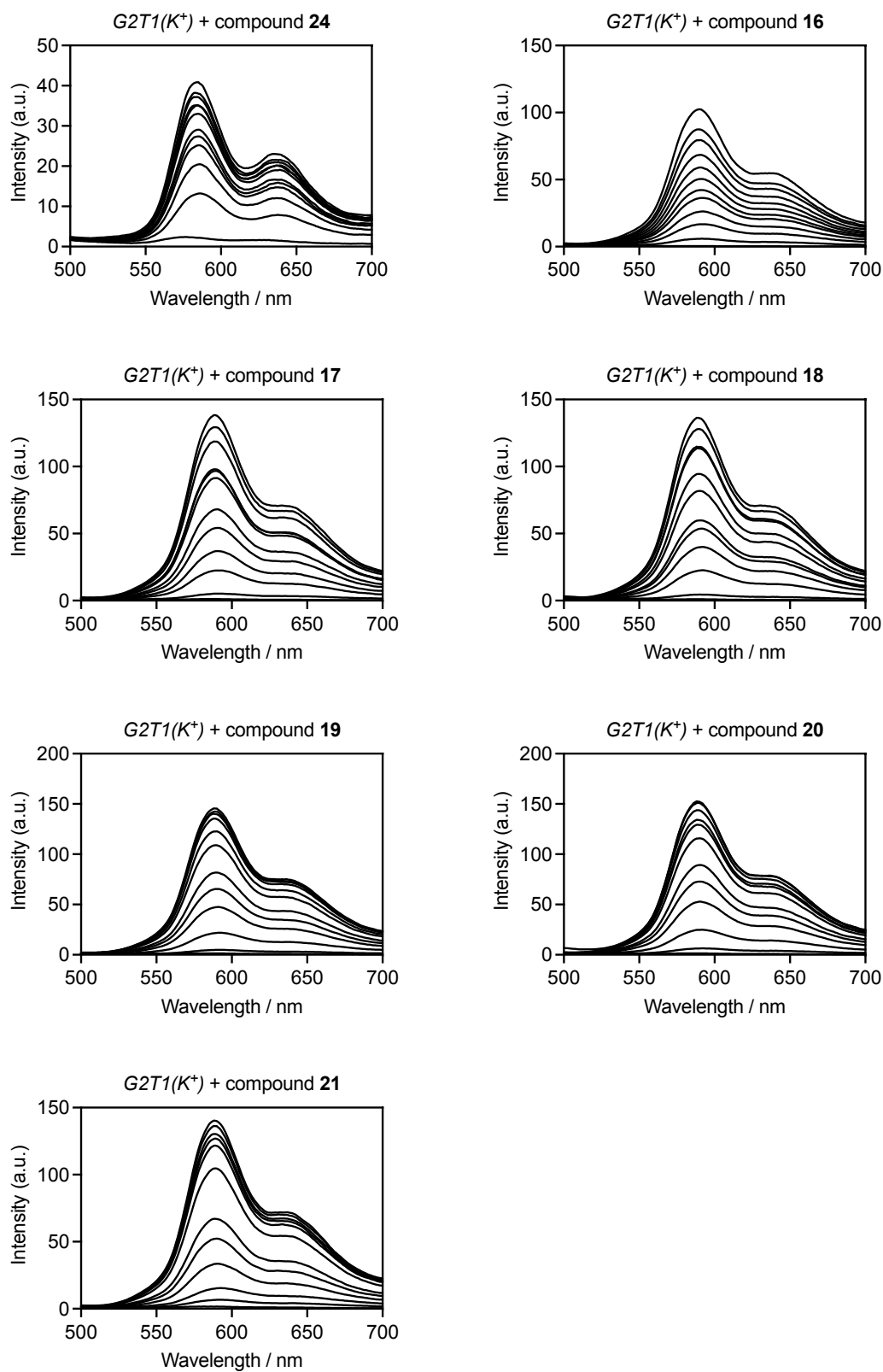


Figure A.101 Emission titrations of Pt^{II}-salphen with G2T1(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0-10 μ M.

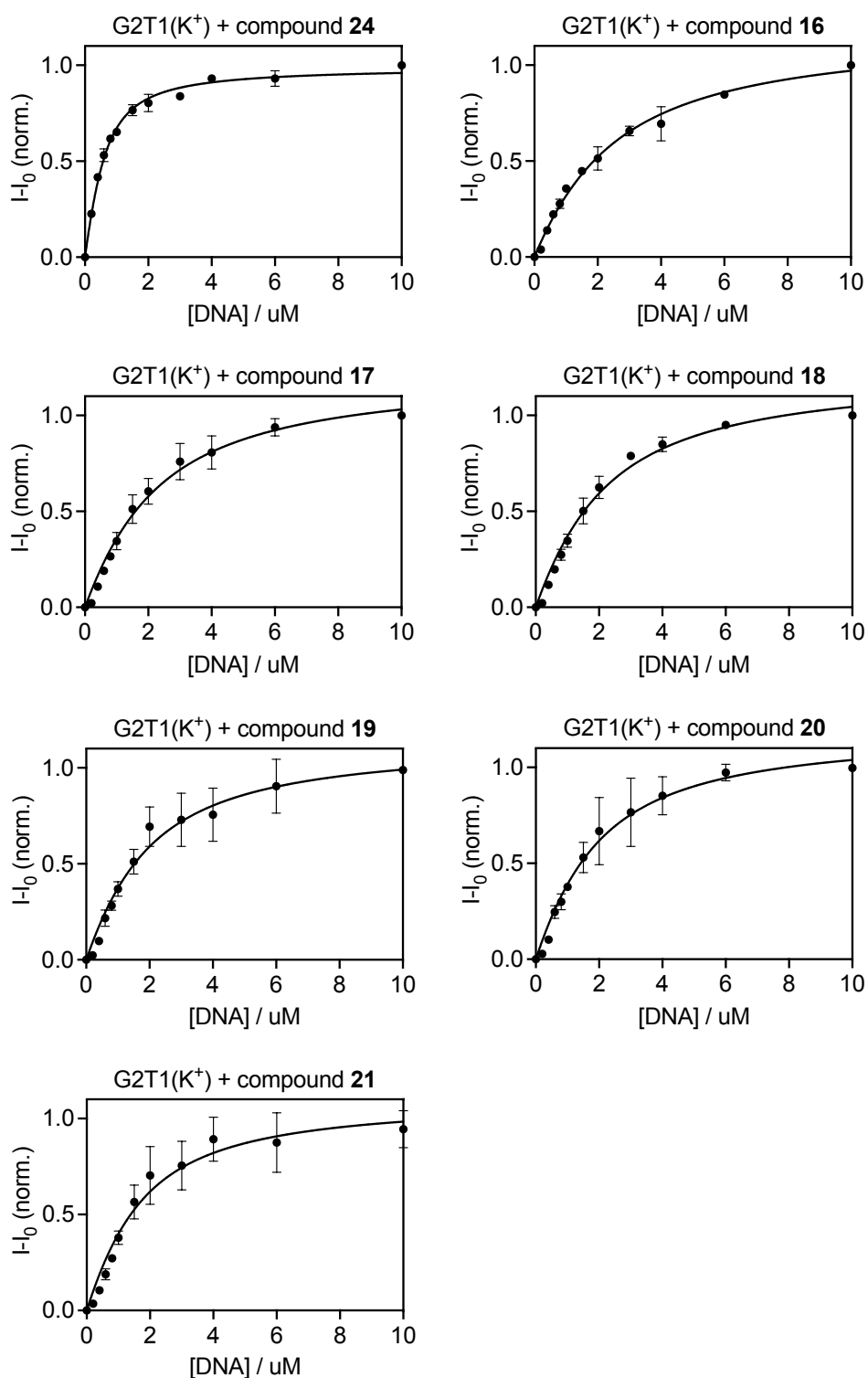


Figure A.102 Fitted data for emission titrations of Pt^{II}-salphens with G2T1(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

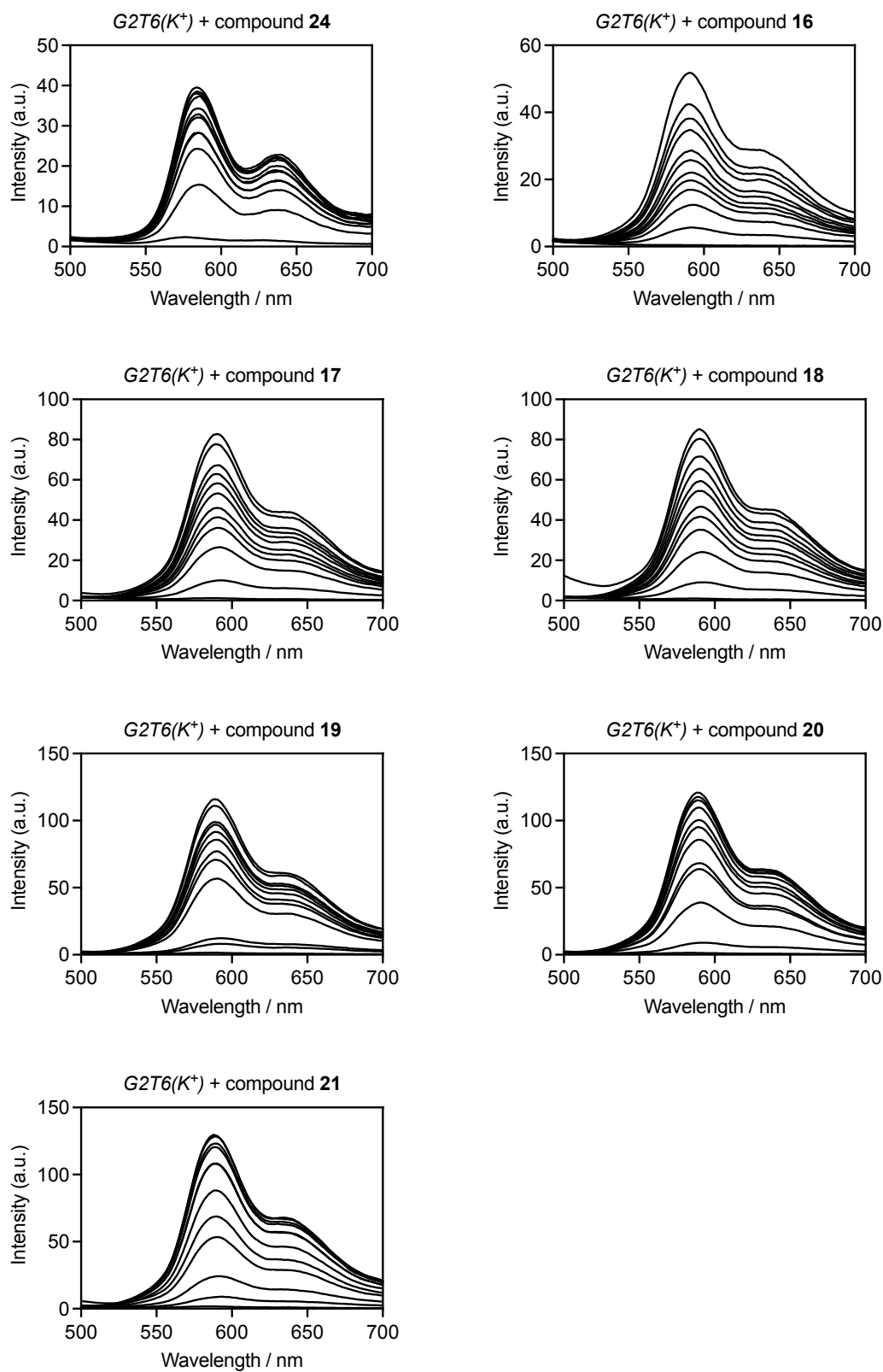


Figure A.103 Emission titrations of Pt^{II}-salphen with G2T6(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0-10 μ M.

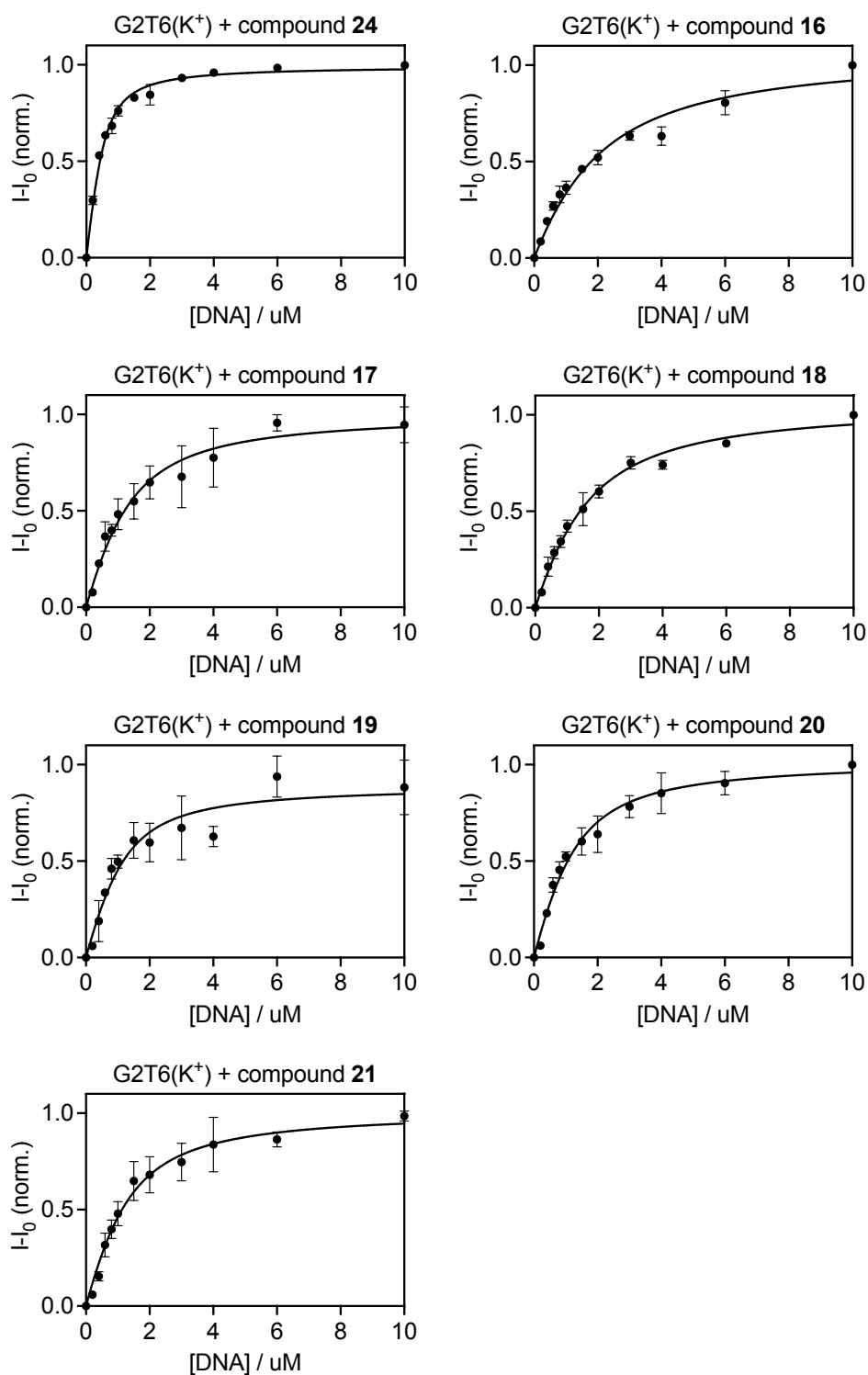


Figure A.104 Fitted data for emission titrations of Pt^{II}-salphens with G2T6(K⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

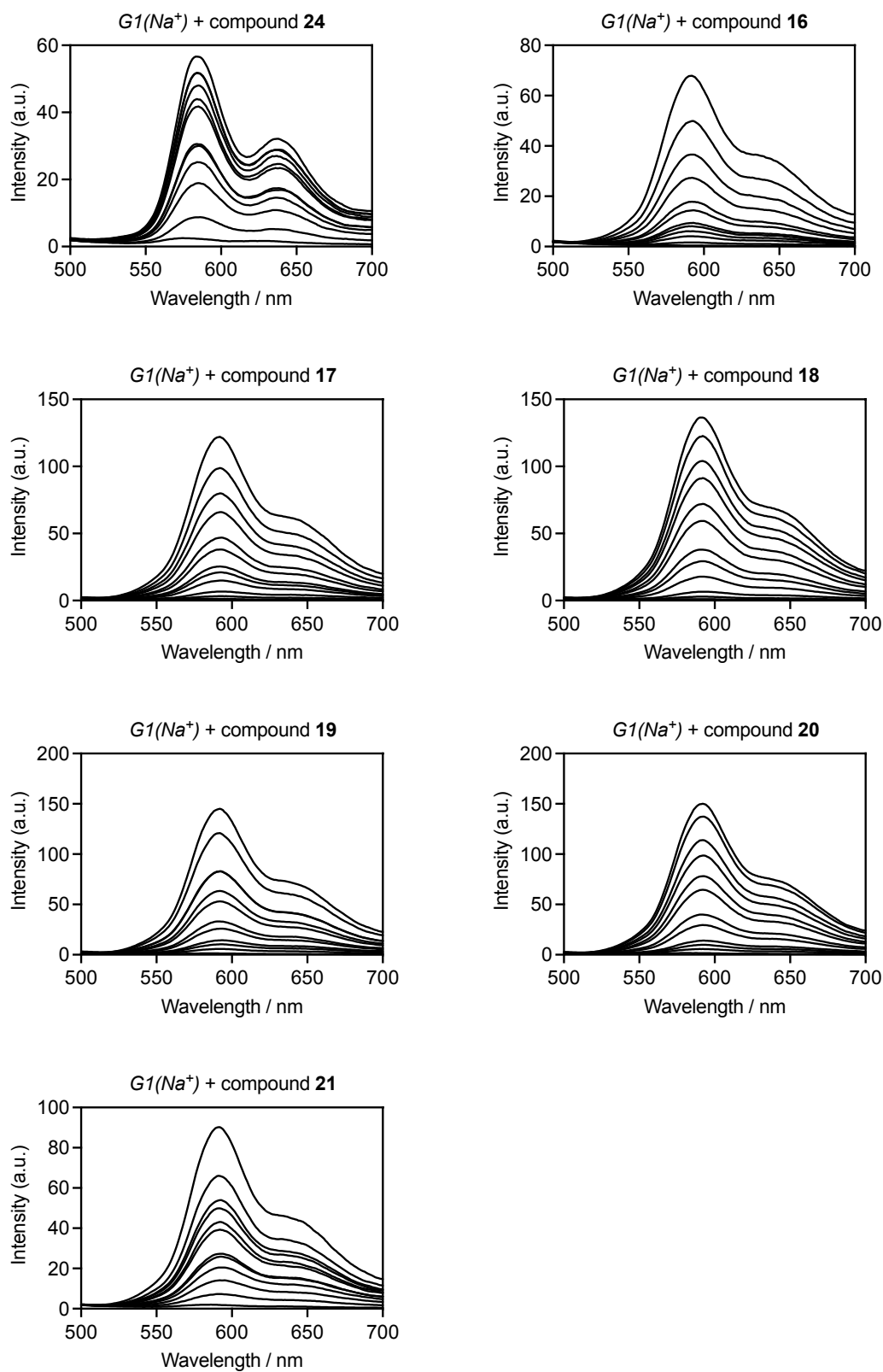


Figure A.105 Emission titrations of Pt^{II}-salphen with G1(Na⁺) DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0-10 μ M.

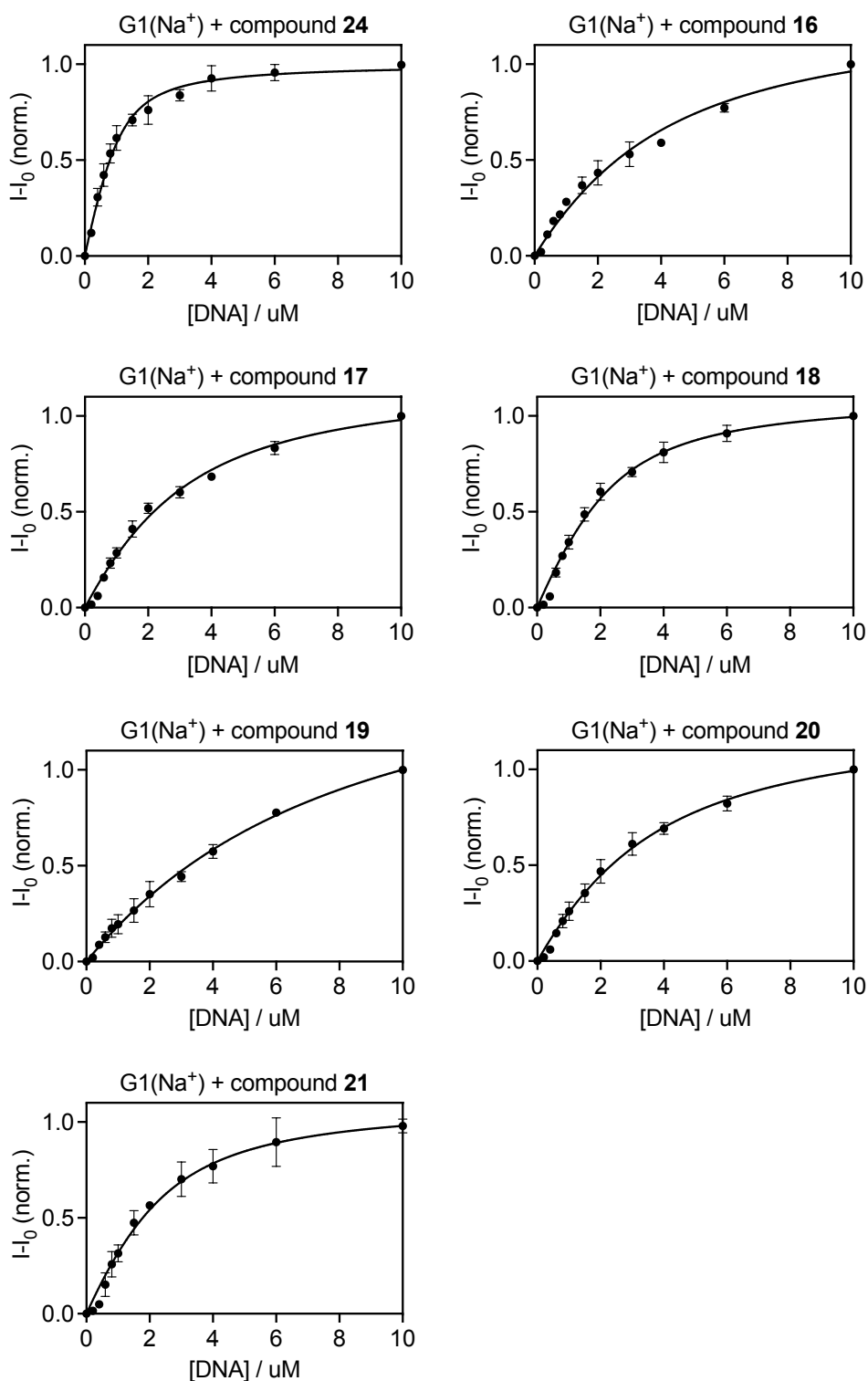


Figure A.106 Fitted data for emission titrations of Pt^{II}-salphens with G1(Na⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

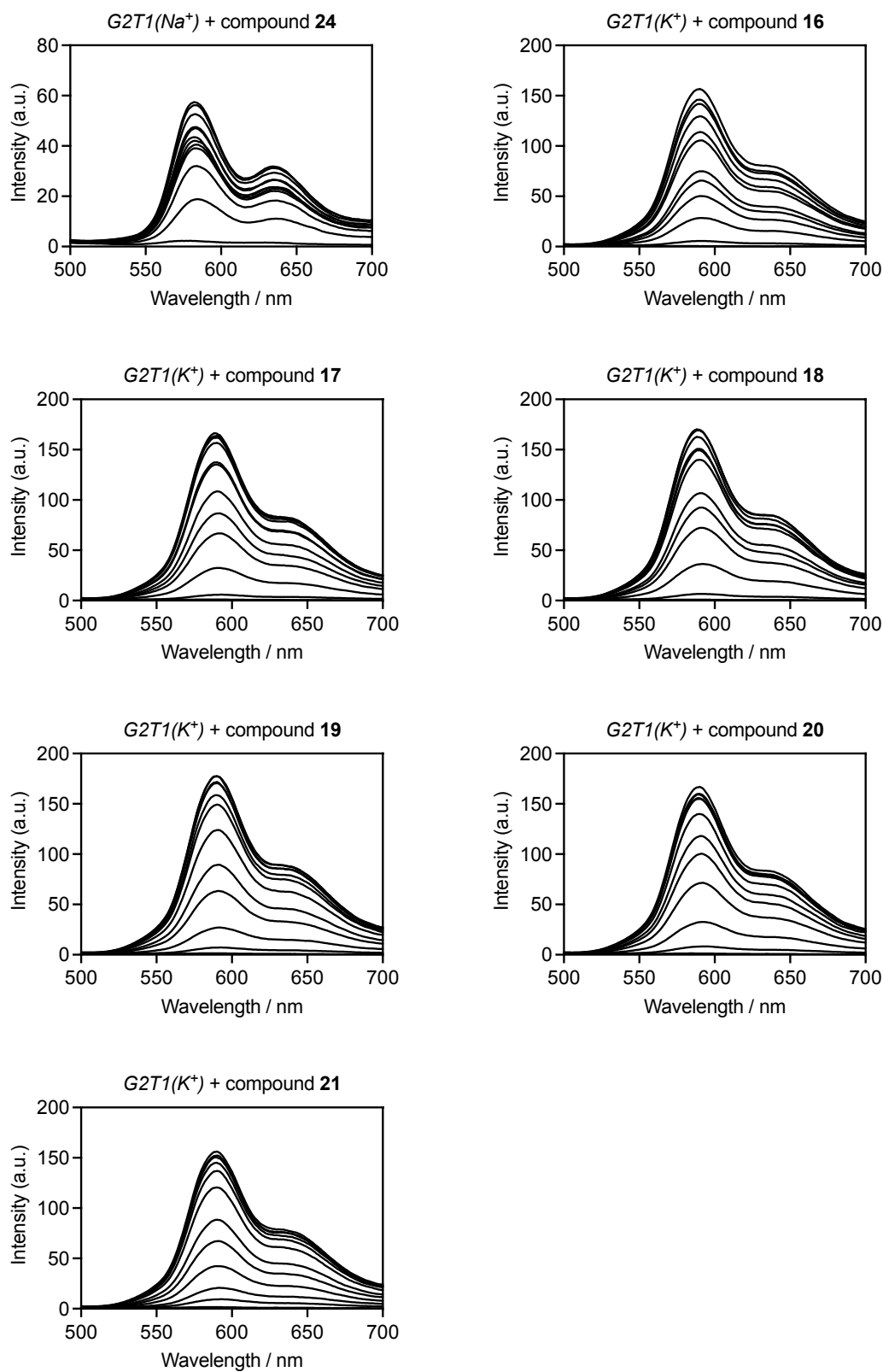


Figure A.107 Emission titrations of Pt^{II}-salphenes with G2T1(Na⁺) DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0–10 μ M.

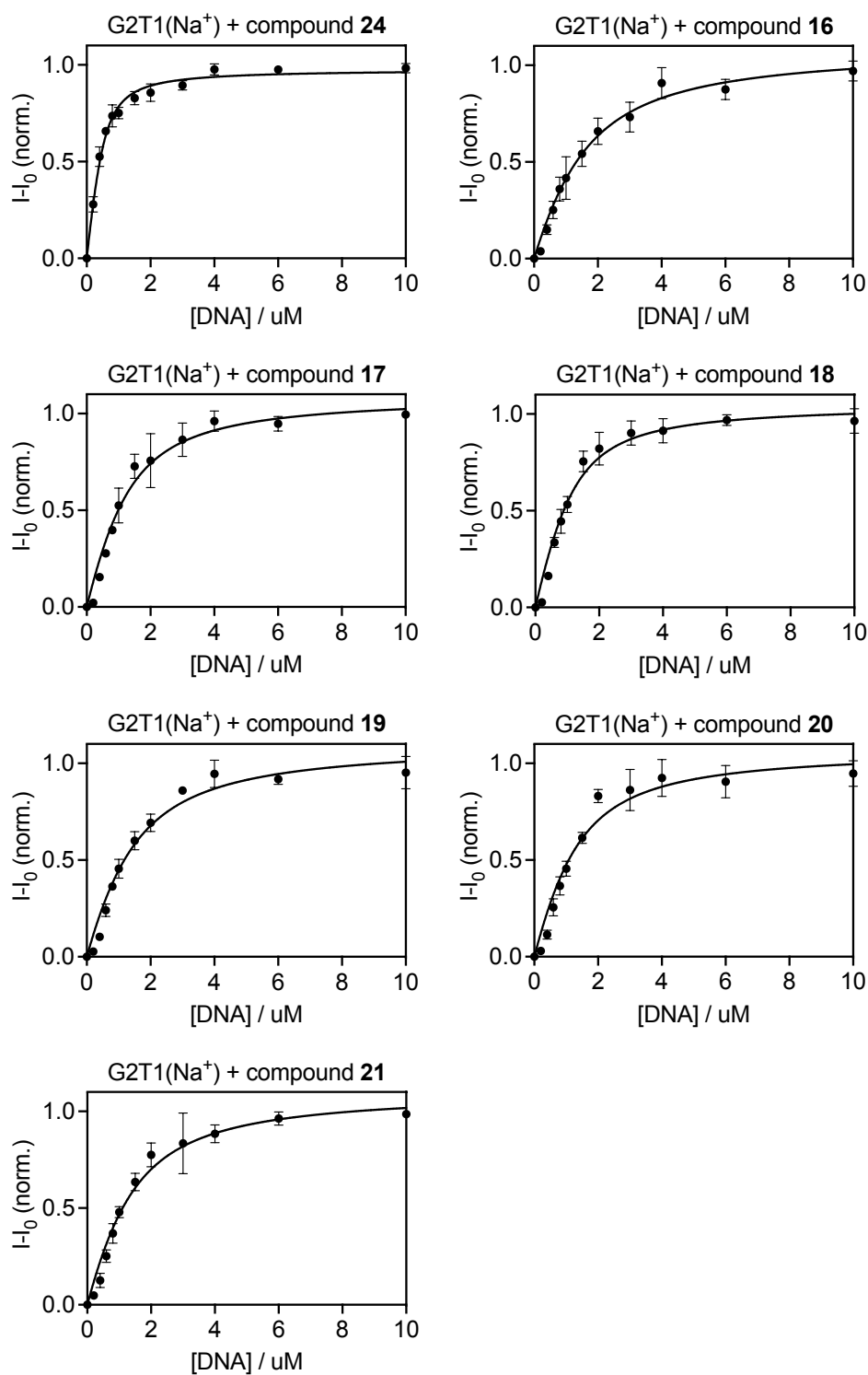


Figure A.108 Fitted data for emission titrations of Pt^{II} -salphens with $\text{G2T1}(\text{Na}^+)$ DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

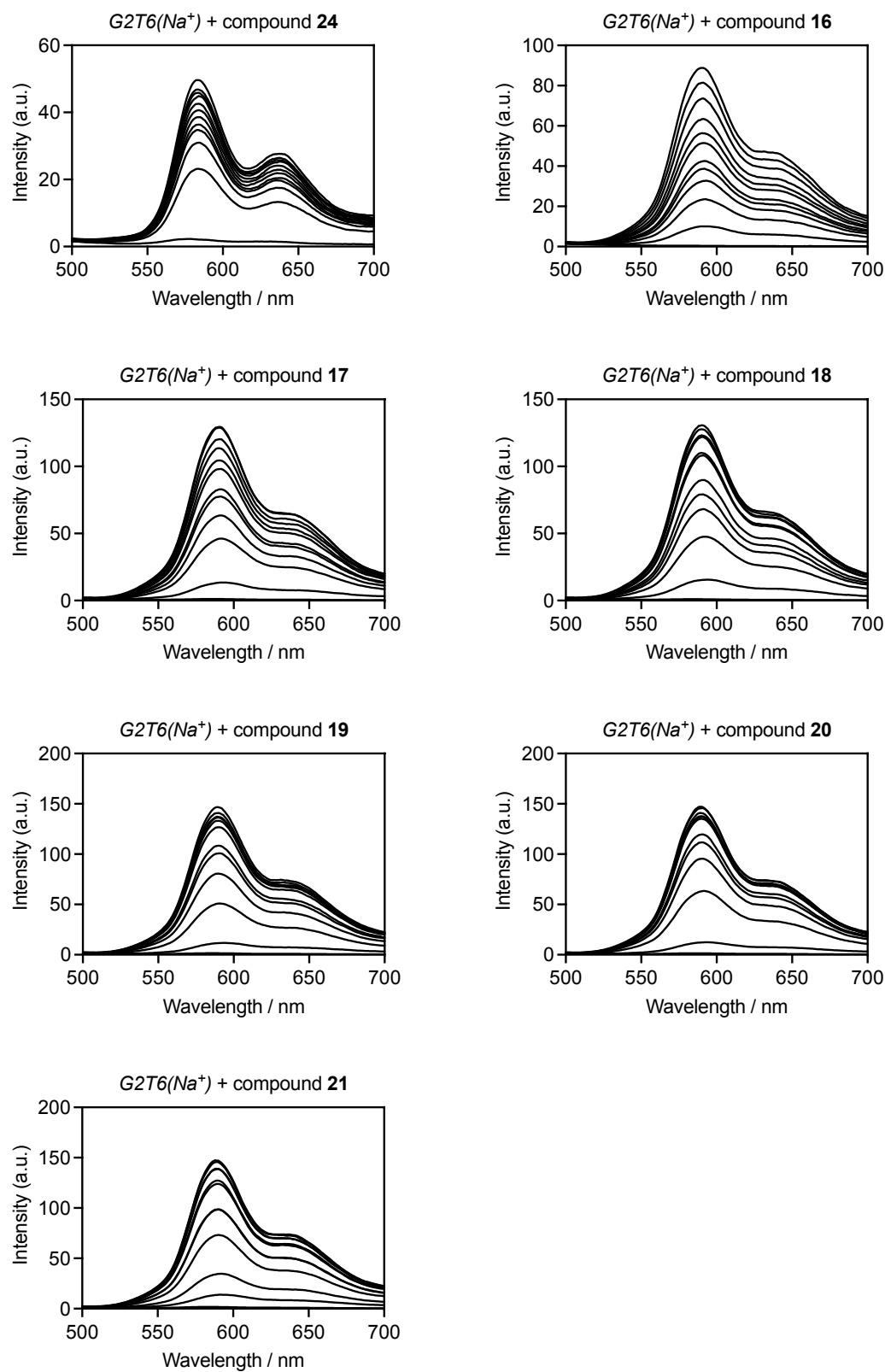


Figure A.109 Emission titrations of Pt^{II}-salphens with G2T6(Na⁺) DNA in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μ M, [DNA] = 0–10 μ M.

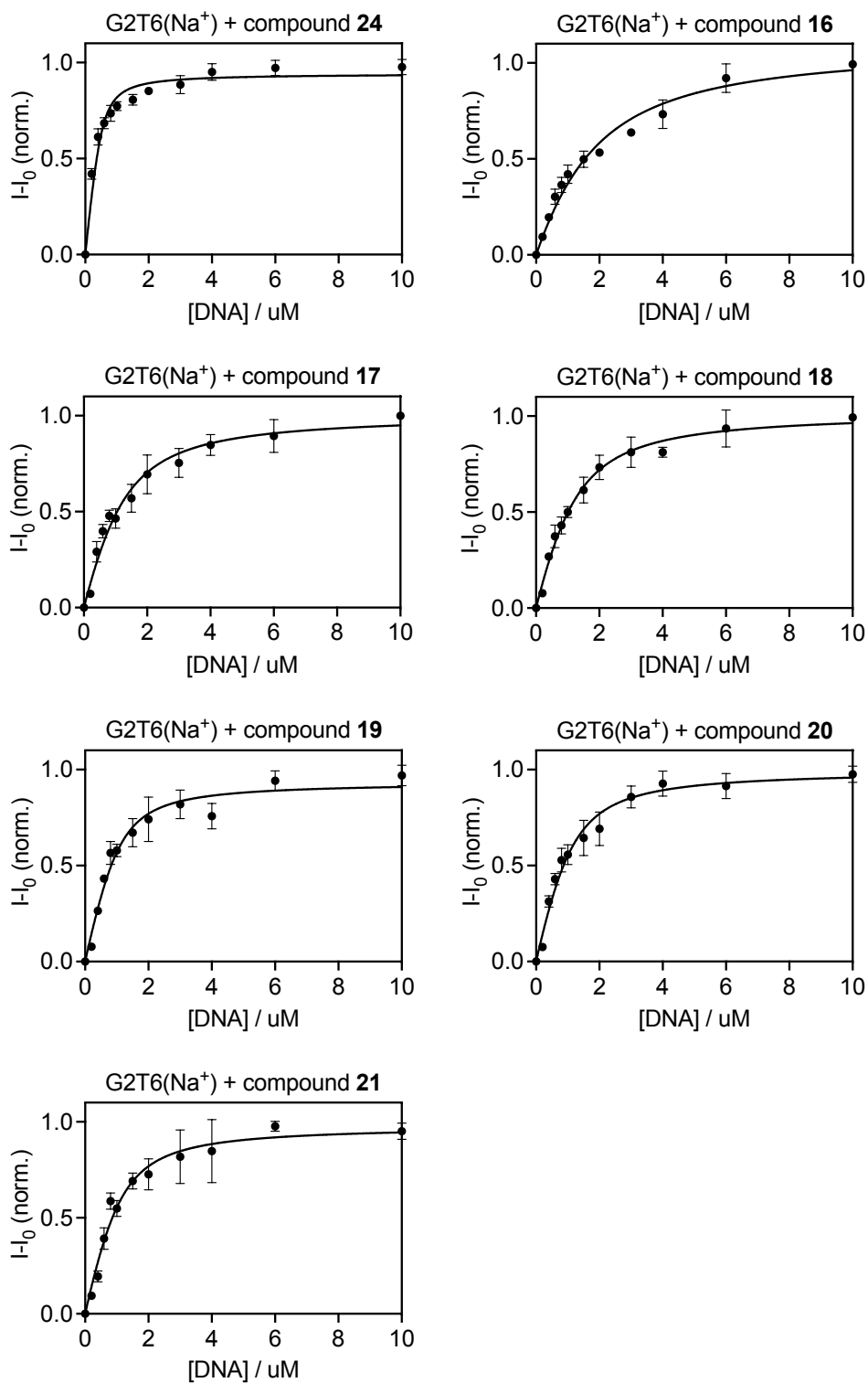


Figure A.110 Fitted data for emission titrations of Pt^{II}-salphens with G2T6(Na⁺) DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0-10 μM .

A.5.2 Chapter 3

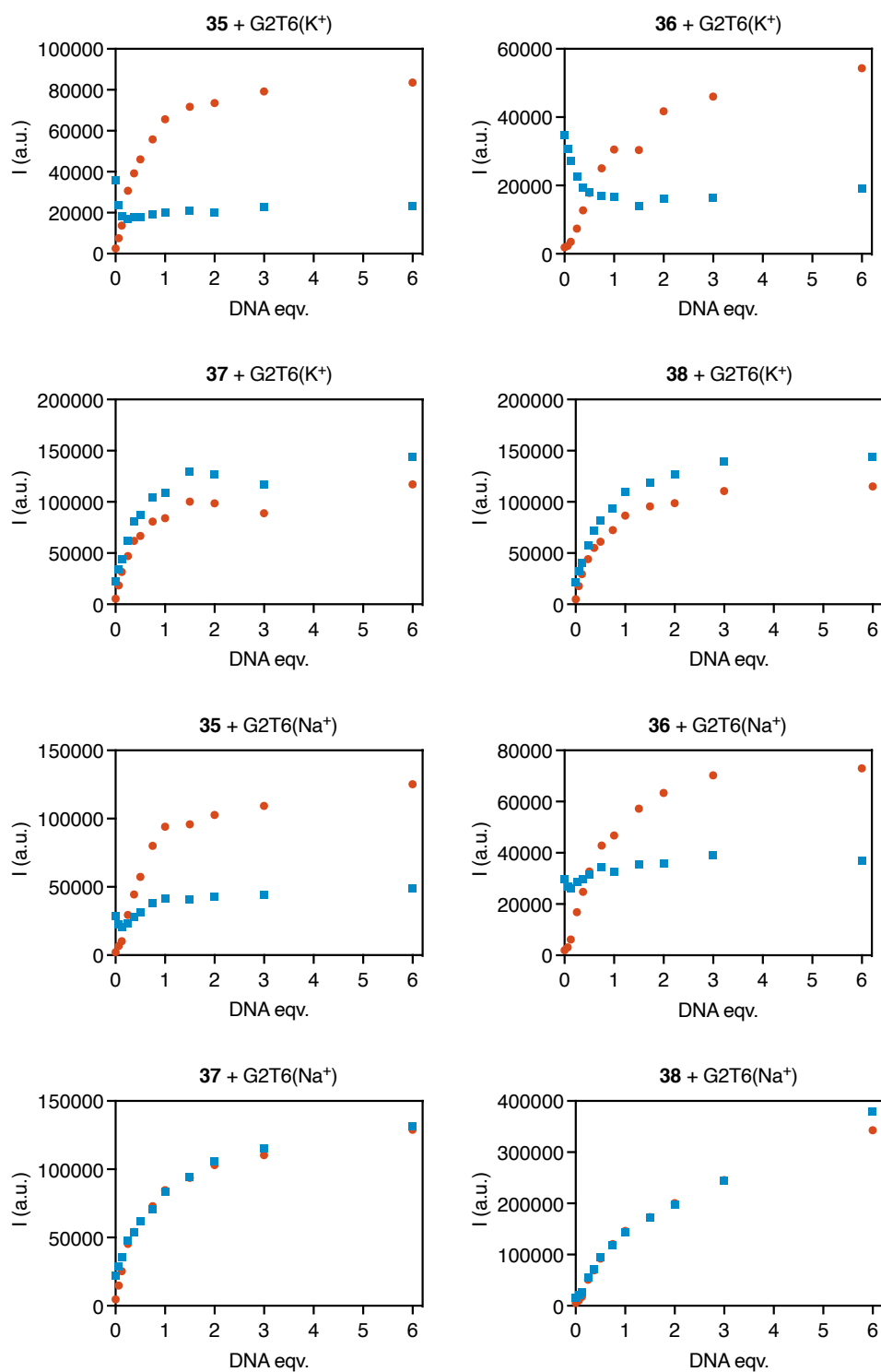


Figure A.111 Raw data titration plots of compounds **35**–**38** with G2T6 G4 DNA in K^+ and Na^+ buffers. Pt^{II} -salphen, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 590$ nm; ADOTA, $\lambda_{ex} = 560$ nm, $\lambda_{em} = 600$ nm). [ligand] = 2 μ M, [DNA] = 0–12 μ M.

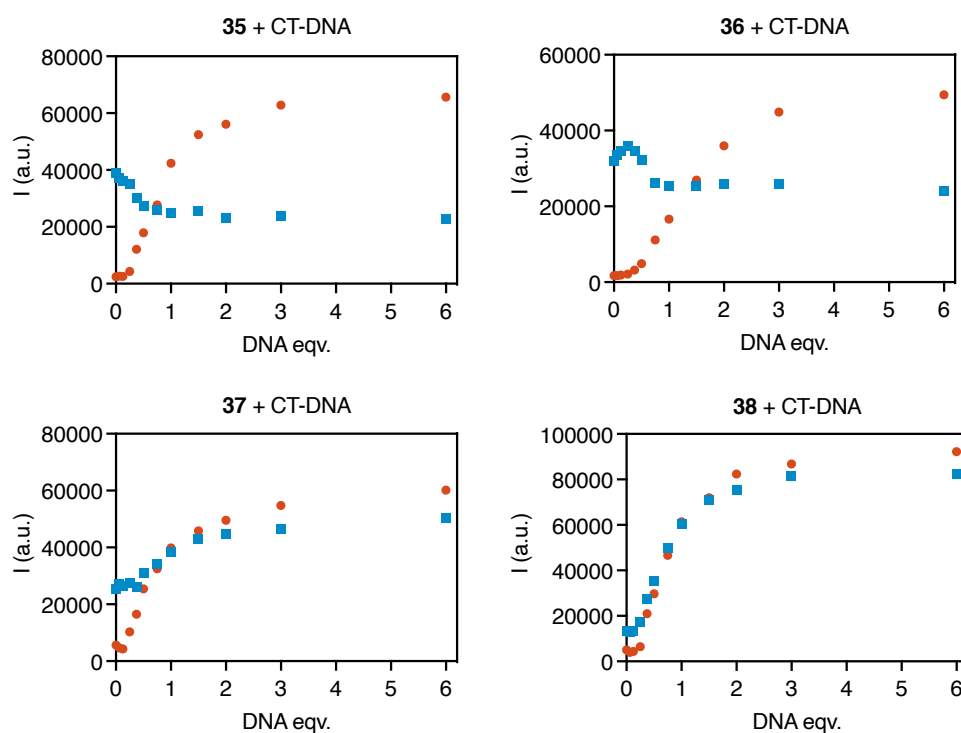


Figure A.112 Raw data titration plots of compounds 35–38 with CT-DNA in K^+ and Na^+ buffers. Pt^{II} -salphen, $\lambda_{ex} = 375$ nm, $\lambda_{em} = 590$ nm; ADOTA, $\lambda_{ex} = 560$ nm, $\lambda_{em} = 600$ nm). $[ligand] = 2 \mu M$, $[DNA] = 0-12 \mu M$.

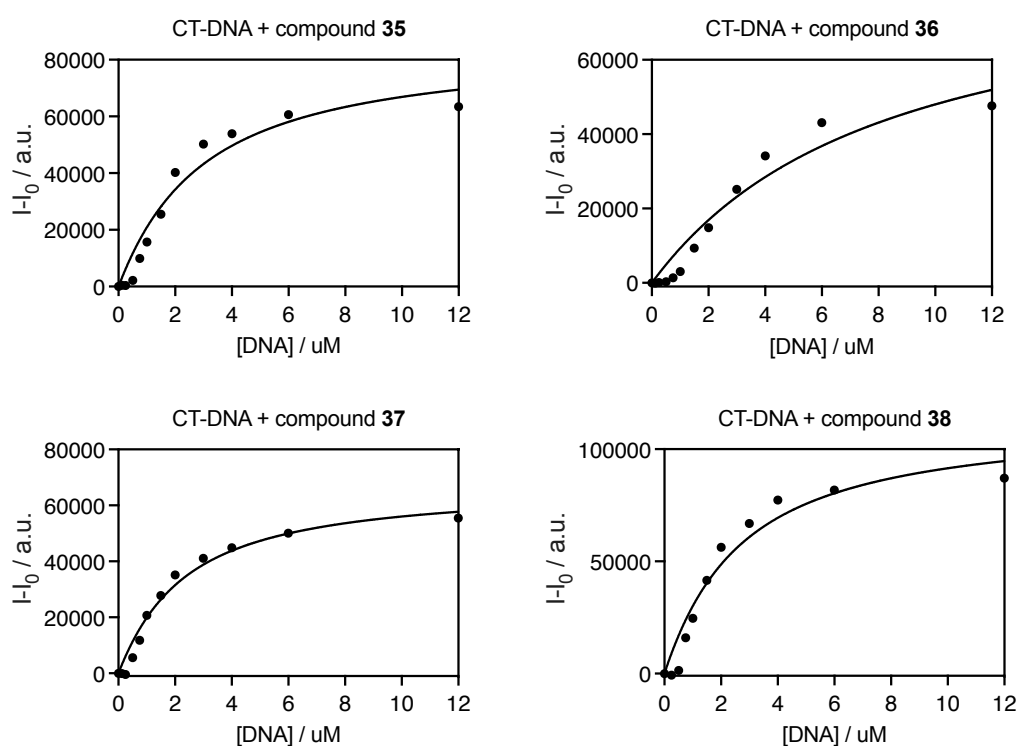


Figure A.113 Fitted data for emission titrations of compounds **35–38** with CT-DNA in 100 mM KCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0–12 μM .

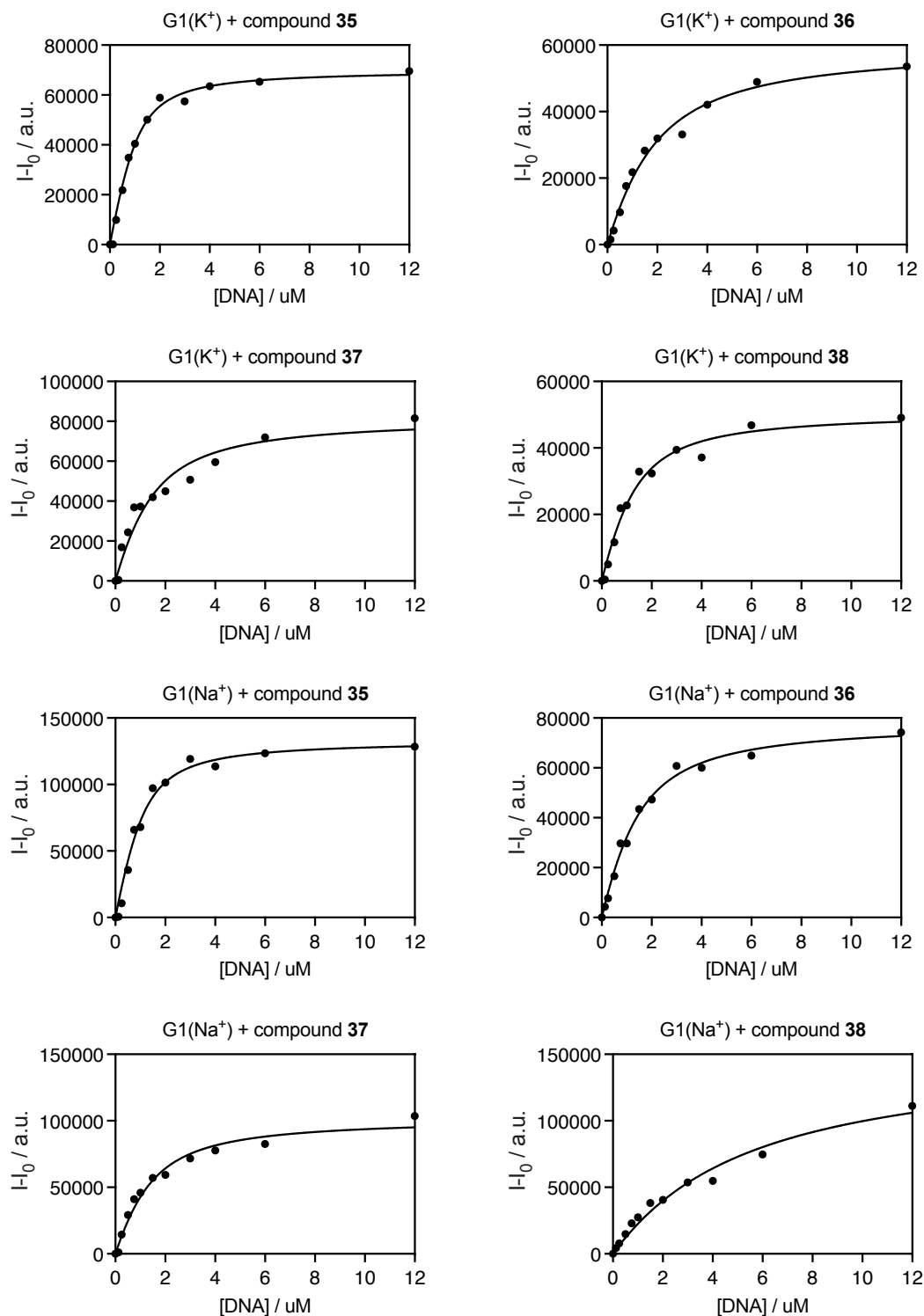


Figure A.114 Fitted data for emission titrations of compounds **35–38** with G1(K^+) DNA in 100 mM KCl and G1(Na^+) in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0–12 μM .

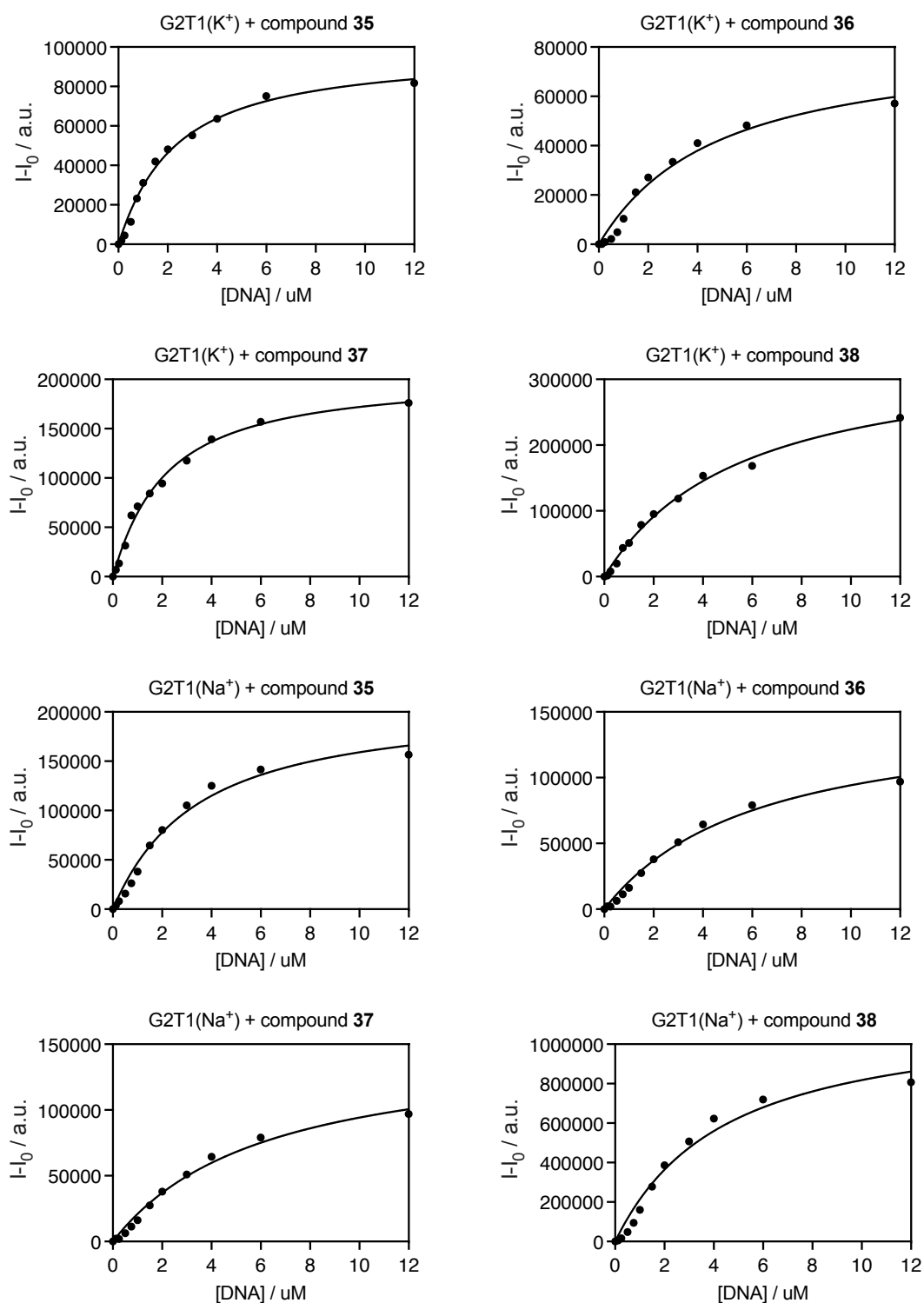


Figure A.115 Fitted data for emission titrations of compounds **35–38** with G2T1(K^+) DNA in 100 mM KCl and G2T1(Na^+) in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0–12 μM .

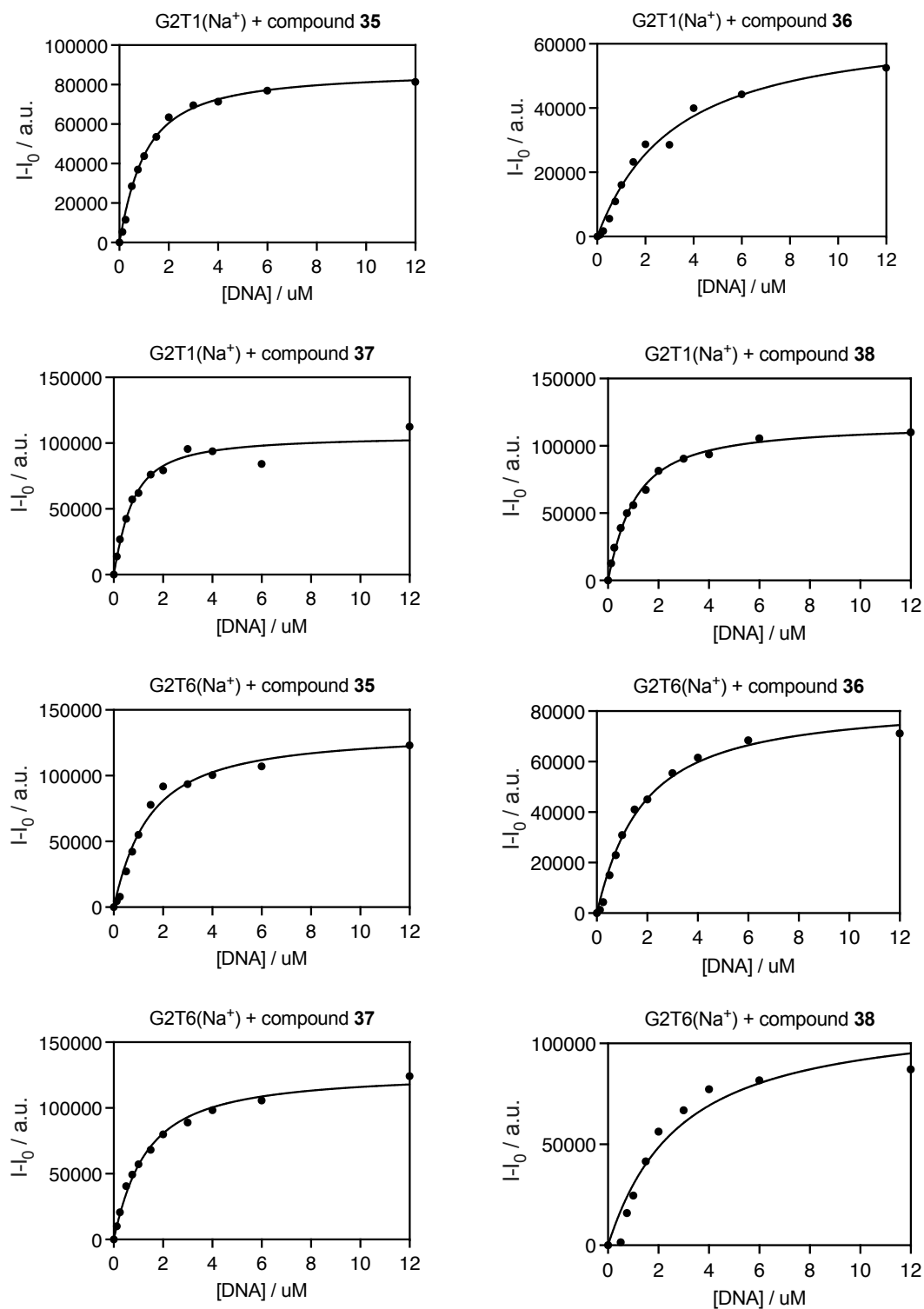


Figure A.116 Fitted data for emission titrations of compounds **35–38** with G2T6(K^+) DNA in 100 mM KCl and G2T6(Na^+) in 100 mM NaCl, 10 mM Li cacodylate, pH 7.3. [ligand] = 2 μM , [DNA] = 0–12 μM .

A.5.3 Chapter 4

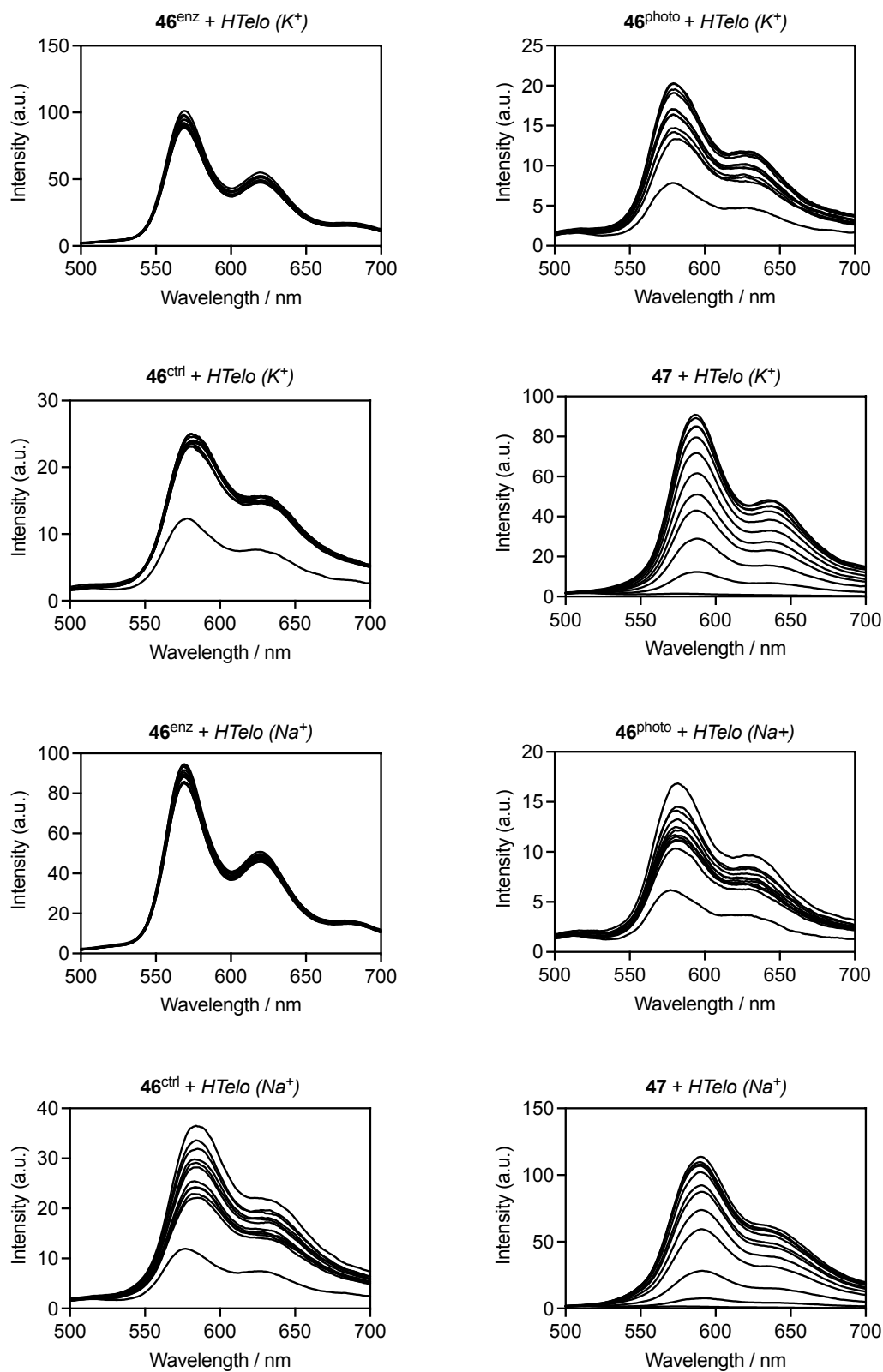


Figure A.117 Rotaxane emission titrations. [ligand] = 2 μ M, [DNA] = 0-10 μ M.

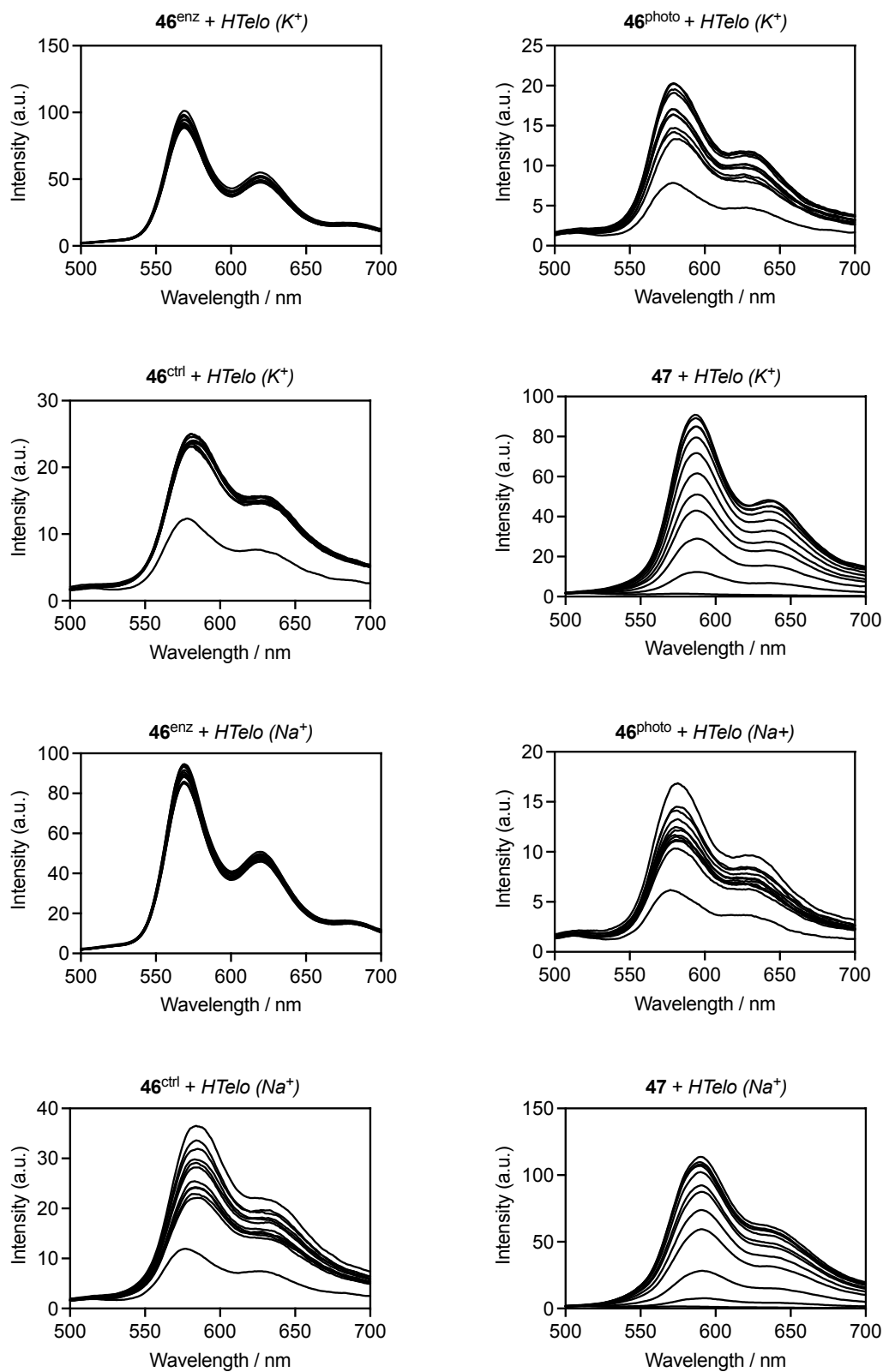


Figure A.118 Rotaxane emission titrations. [ligand] = 2 μ M, [DNA] = 0-10 μ M.

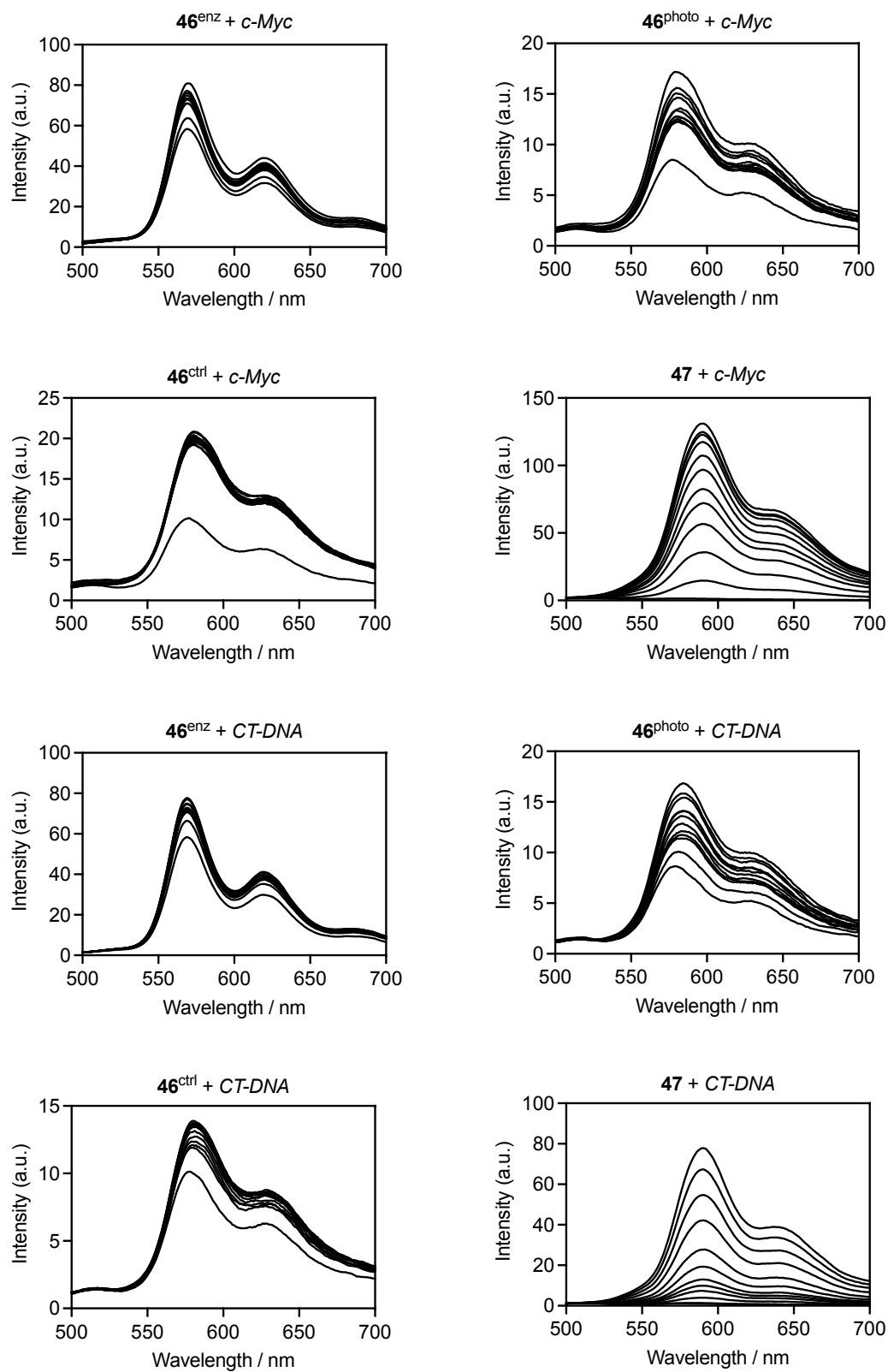


Figure A.119 Rotaxane emission titrations. [ligand] = 2 μM , [DNA] = 0-10 μM .

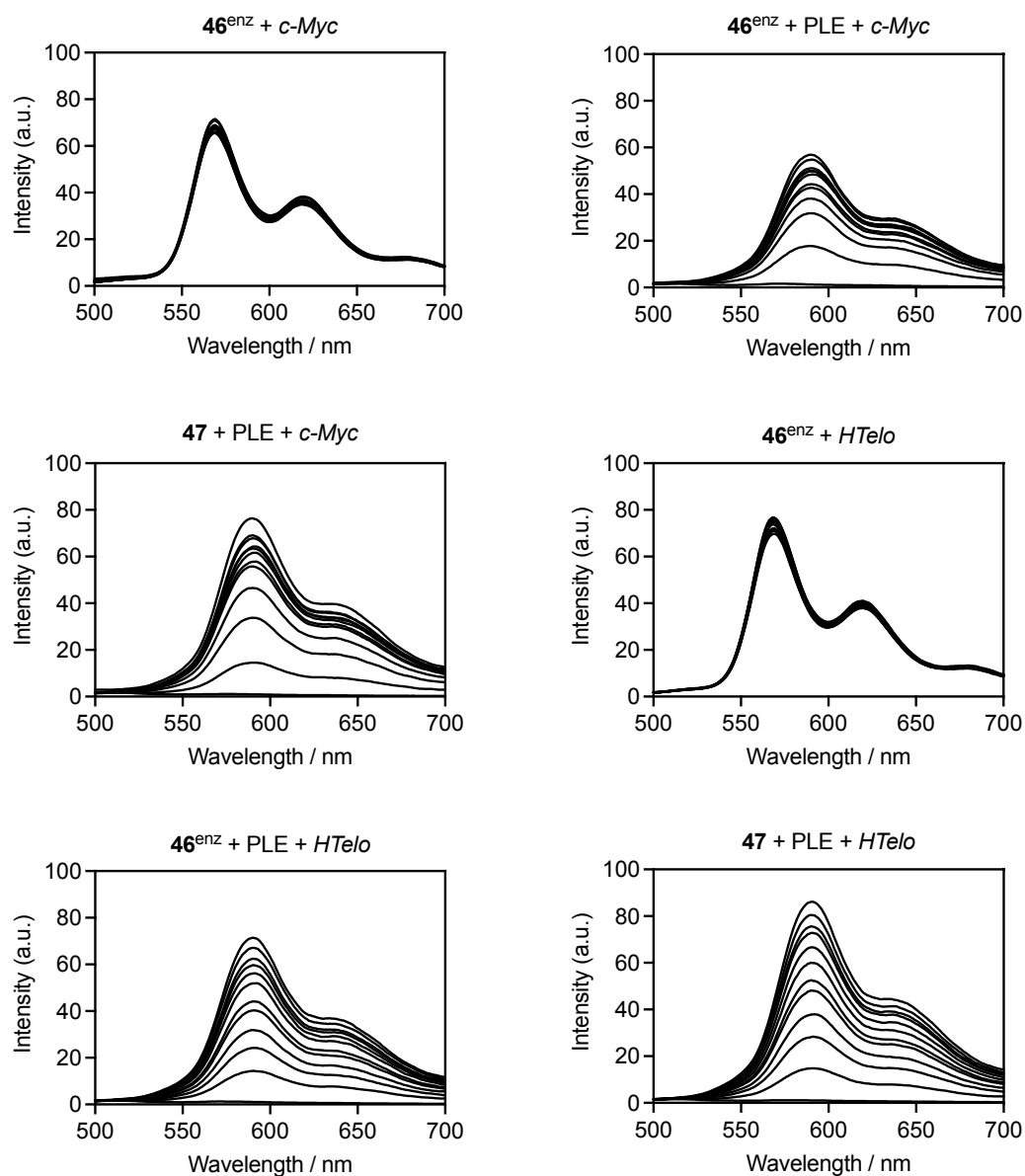


Figure A.120 Rotaxane emission titrations after treatment with porcine liver esterase. [lig- and] = 2 μM , [DNA] = 0-10 μM .

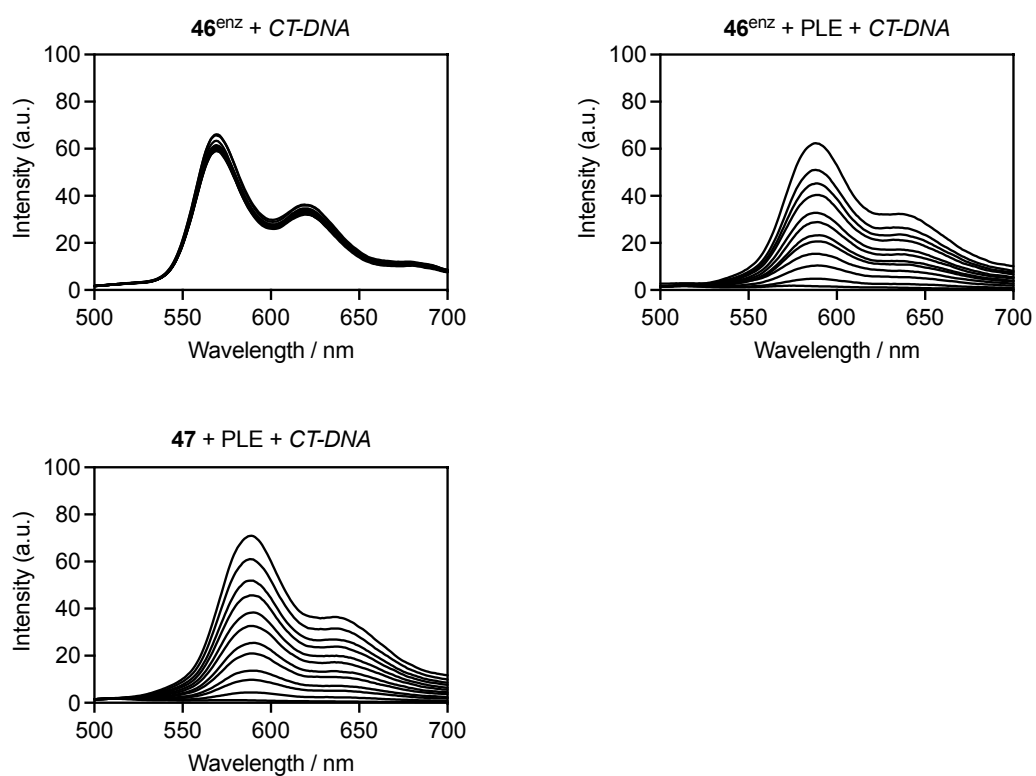


Figure A.121 Rotaxane emission titrations after treatment with porcine liver esterase. [lig- and] = 2 μ M, [DNA] = 0-10 μ M.

A.6 Rotaxane stability experiments

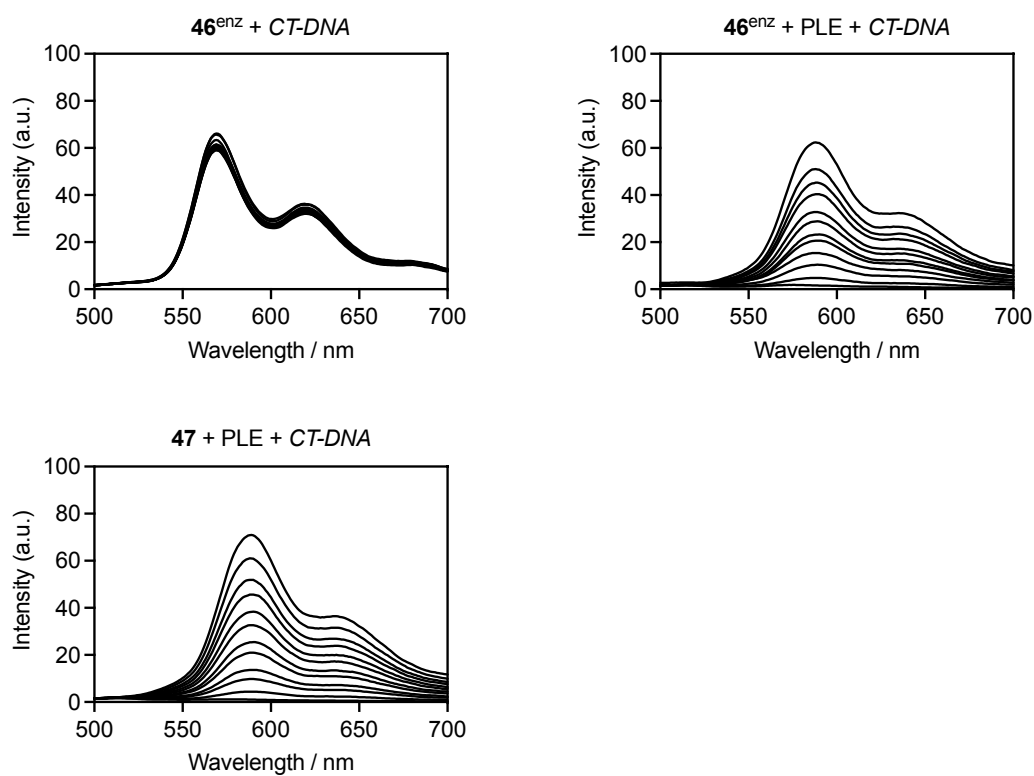


Figure A.122 Rotaxane stability in PBS buffer at 37 °C after 24 hours.

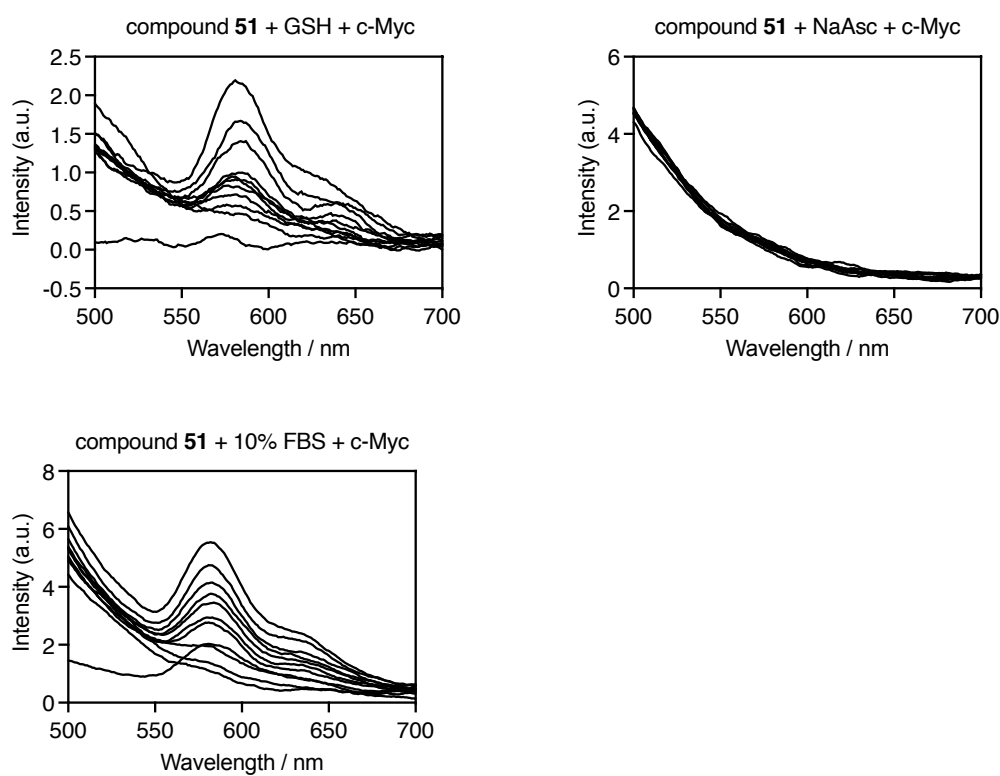
A.7 Pt^{IV} -salphen stability experiments

Figure A.123 Stability of Pt^{IV} -salphen 51 in the presence of various reducing agents.