

The design and synthesis of DNA binding compounds targeting DNA junctions

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Declaration

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Abstract

The uncontrolled proliferation of cancer cells leads to the upregulation of DNA repair pathways, highlighting this process as an interesting target for cancer therapy. DNA junctions are secondary structures that form during DNA repair. Four-way junctions assemble during homologous recombination, a major DNA repair pathway responsible for the repair of double-strand DNA breaks. *N,N'*-Dimethylaminoethyl 9-aminoacridine-4-carboxamide dimers linked through a flexible methylene chain are cytotoxic compounds that can bind to DNA through intercalation. They have also been shown to bind to four-way junctions via a novel mechanism, although this imparted little selectivity over double-stranded DNA.

In this thesis, the design and synthesis of twelve acridine-based dimeric compounds is presented, including six novel small molecules designed with restricted flexibility to bind across four-way junctions via duplex-to-duplex DNA crosslinking. DNA binding to duplex DNA and four-way junctions was assessed through a displacement assay and electrophoretic mobility shift assays. The effect on the stability of the 4WJ upon ligand binding was investigated through melting experiments, further determining 4WJ selectivity in the presence of secondary DNA structures.

Furthermore, the synthesis of two novel acridine trimers is reported, targeting three-way DNA junctions, designed to bind to multiple sites of the junction simultaneously. Gel electrophoresis studies showed that the compounds can promote the formation of a three-way junction at concentrations as low as 1.56 μM , whereas they have no effect on four-way junctions at this concentration. Additionally, the trimers cause a significant increase in the stability of a three-way junction upon binding ($\Delta T_m = 30\text{ }^\circ\text{C}$, 10 μM).

The designed acridine dimers and trimers were evaluated for cellular efficacy in leukaemia, melanoma and pancreatic cancer cell lines. A cell assay indicated synergy between the acridine dimers and DNA repair inhibitors in cancer cells, offering a potential novel therapeutic approach in cancer treatment.

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Abbreviations

3WJ	Three-way junction
4WJ	Four-way junction
Å	Angstrom
A	Adenine
ALT	Alternative-Lengthening Pathway
Aq	Aqueous
AuNPs	Gold nanoparticles
Boc	Tert-butyloxycarbonyl
BMH	Bismaleimidohehexane
C	Cytosine
ct-DNA	Calf-thymus DNA
CuAAC	Copper(I)-catalysed azide alkyne cycloaddition
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand break
<i>E.coli</i>	<i>Escherichia coli</i>
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
Em	Emission
Eq.	Equivalent
EMSA	Electrophoretic mobility shift assay
Et₂O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
Ex	Excitation

FAM	6-carboxyfluorescein
FBS	Foetal bovine serum
FRET	Förster resonance energy transfer
G	Guanine
G4	G-Quadruplex
HJ	Holliday junction
HPLC	High-performance liquid chromatography
HR	Homologous recombination
HRMS	High-resolution mass spectrometry
iM	i-motif
IR	Infrared spectroscopy
LC-MS	Liquid chromatography – Mass spectrometry
LRMS	Low-resolution mass spectrometry
MeOH	Methanol
MTS	3-[4,5,dimethylthiazol-2-yl]-5-[3-carboxymethoxy-phenyl]-2-[4-sulfophenyl]-2H tetrazolium
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PPA	Polyphosphoric acid
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute 1640 Medium
RT	Room temperature
T	Thymine
T_m	Melting temperature
TAMRA	Tetramethyl-6-carboxyrhodamine
TBE	Tris/Borate/EDTA
TLC	Thin layer chromatography
U	Uracil
UV	Ultra violet

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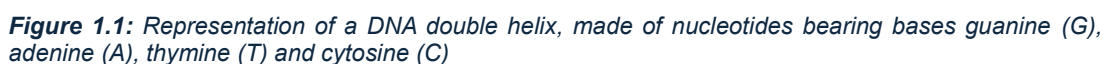
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Chapter 1

Introduction

Genetic information is stored in molecules of deoxyribonucleic acid (DNA). DNA consists of two polynucleotide chains, coiled around each other, composed of four types of nucleotides.¹ Each nucleotide contains a five-carbon sugar attached to a phosphate group, linked to a nitrogen-bearing ring structure via a glycosidic bond at the sugar 1' position, called a base (**Figure 1.1**). The four types of bases are adenine (A), thymine (T), cytosine (C) and guanine (G), and the order of these bases in the DNA defines the genetic code of a cell.¹ The bases are split into two groups based on their structures, T and C are described as pyrimidines (py), whilst A and G are purines (pu). The three-dimensional structure of DNA was first described as a double helix in 1953 by James Watson and Francis Crick, based on experimental observations obtained by various scientists, including Rosalind Franklin and Maurice Wilkins.²⁻⁴ A right-handed helix was presented, with a sugar-phosphate backbone forming the exterior, held together by hydrogen bonded bases. As seen in **Figure 1.1**, the four bases contain several hydrogen bond donors and acceptors, allowing them to form stable base pairs, with adenine always pairing with thymine, and guanine with cytosine. The latter is the strongest pair, as three hydrogen-bonds hold the bases together compared to A-T, held together by two. Therefore, in a double helix, the two DNA chains are described as complementary, allowing for the sequence of one to be used to determine the sequence of the other.



Nucleotides are linked together with phosphodiester bonds that form between the 5'-hydroxyl group of one sugar and the 3'-hydroxyl group of the next sugar, presented in **Figure 1.1**. The polarity of each DNA strand is determined by the chemical nature of the first and last nucleotide of a chain.⁵ The beginning of the chain is defined as the 5'-end, due to the exposed free phosphate group bound to the 5'-carbon of the first nucleotide. The opposite extremity is the 3'-end, as it terminates with a free hydroxyl group of the 3' carbon of the last nucleotide. The assembly of two DNA strands into a double helix is only permitted when oriented in an antiparallel conformation, the 5'-terminal nucleotide of one chain always opposite the 3'-terminal nucleotide of the other.

1.2 Double helical forms of DNA

1.2.1 B-form DNA

The canonical right-handed structure described in **Section 1.1** is the most abundant form of DNA helix found in living cells under physiological conditions and is defined as B-DNA.¹ The two DNA chains wrap around the same axis, causing the base pairs to stack, forming one complete turn every 10 base pairs. The geometry of the helix leads to the formation of two grooves that run diagonally through the length of the helix, a 12 Å wide major groove, located opposite a 6 Å wide minor groove.⁶ DNA can exist in two additional helical forms, A-DNA and Z-DNA.

1.2.2 A-form DNA

The X-ray diffraction experiments carried out by Franklin and Gosling that led to the discovery of the B-DNA structure, also uncovered a second helical DNA structure, known as A-DNA.² Significant differences are noted in its geometry. Whilst A-DNA is also a right-handed helix, it is a wider and more compact structure. In B-DNA, the sugars located in the backbone are in the C2' endo-conformation, with the C2' atom facing upwards in the same direction as the base. However, in A-DNA the sugar is arranged in a C3' endo-conformation, with the C2' and C3' atoms facing the opposite way, shown in **Figure 1.2**. Additionally, the vertical spacing between the base pairs is reduced to 2.56 Å, whilst the base pairs are tilted in a 20° angle from the plane perpendicular to the helix axis, containing 11 base pairs per turn. This geometry results in a displaced axis, therefore a shallower major groove and a deeper minor groove, also forming a 3 Å wide hole in the centre of the helix, leading to a less stable complex than B-DNA.

Extensive research into the biological relevance of A-DNA suggest that this conformation may be induced upon binding of specific proteins to DNA, or act as an intermediate in forming distorted DNA conformations required for certain protein-DNA complexes.⁷⁻⁹ Conditions of low humidity favour the A-form DNA, as it exposes more hydrophobic surfaces in comparison to B-DNA.

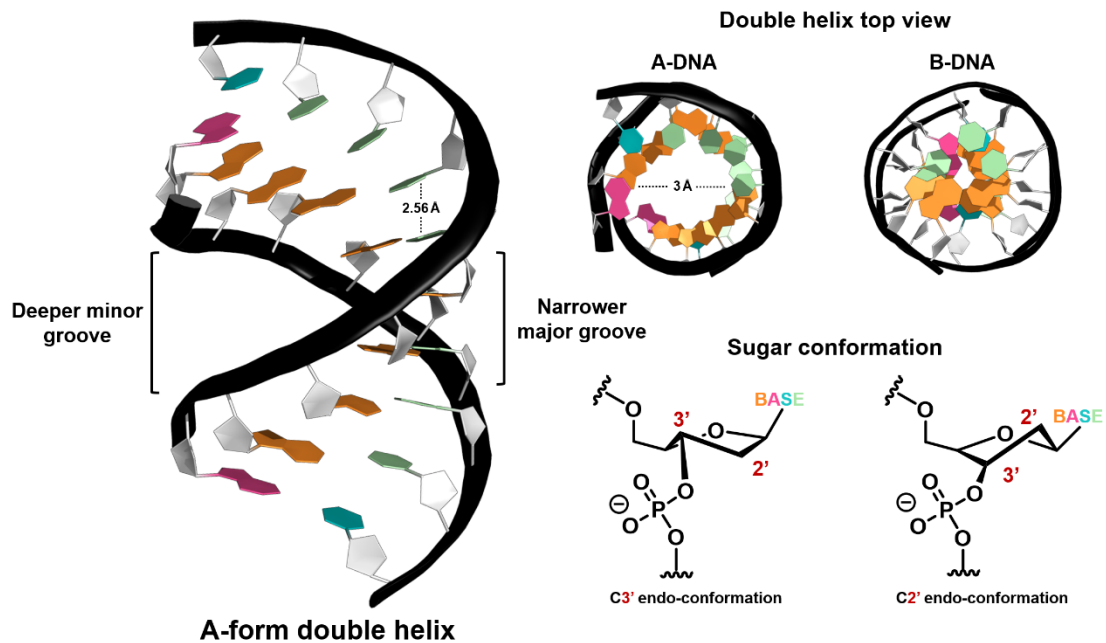


Figure 1.2: Crystal structure of A-form DNA (PDB 440D), the double helix has a characteristic narrower major groove and deeper minor groove compared to the B-DNA. The top view of the helix highlights a 3 Å gap form. Nucleotide sugars in A-DNA are in the C3' endo-conformation whilst B-DNA are in the C2' endo-conformation.

1.2.3 Z-form DNA

A third form of helical DNA was detected in 1967¹⁰, described as B-DNA of inverted, left-handed helicity, later confirmed in 1979 as a third double helical structure, defined as Z-DNA.¹¹ In contrast to A- and B-DNA, the Z-DNA helix is left-handed, characterised by a narrower and more elongated geometry, while maintaining an antiparallel orientation of the two strands, bound through standard Watson-Crick base pairing. The backbone of the helix forms a unique zig-zag pattern, responsible for the structure's name, which is able to form in stretches of alternating purine-pyrimidine bases. The zig-zag motif reflects the alternating conformations adopted by purines and pyrimidines in this architecture. The purines are arranged in the *anti*-conformation and their sugars in C2' endo, similar to B-DNA, whereas the pyrimidines are in *syn*-conformation with their sugars in C3' endo.^{12,13} The altered backbone leads to 12 base pair long turns and an increase to 3.7 Å per base pair, with no major groove forming, only a minor groove (**Figure 1.3**).

The resulting pattern reduces certain distances between phosphate groups, causing greater electrostatic repulsion between the two complementary strands than in B-DNA. Stabilisation is accomplished in high salt environments or in the presence of polyvalent cations, shielding the strands from interphosphate repulsion. Z-DNA forming sequences are not distributed randomly in the genome. A study screening across 137 complete genes, identified 329 Z-DNA forming sequences, with a strong bias towards transcription initiation locations.¹⁴ A direct correlation has been shown between Z-DNA formation and transcriptional activity, suggesting that transcription can promote the Z-DNA conformation, whilst Z-DNA can regulate transcription activity.^{15,16} Extensive research has been carried out to understand the role of Z-DNA in the genome, the structure has been associated with several biological and pathological processes, however further research is required to understand its role.¹⁷

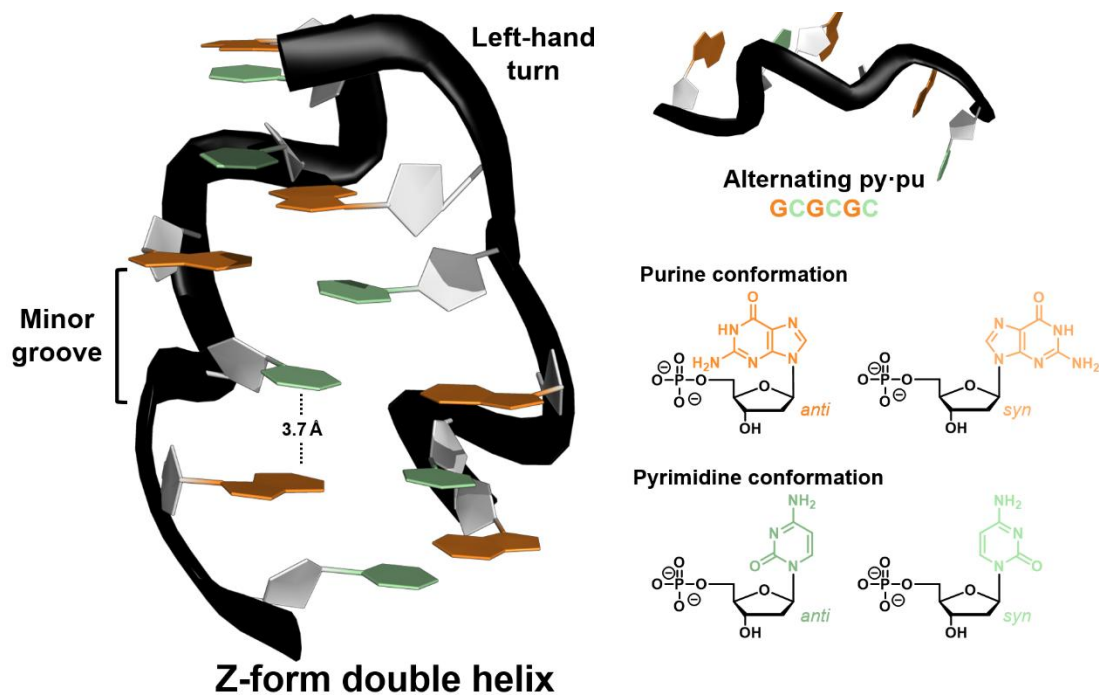


Figure 1.3: Crystal structure (PDB **4R15**) of Z-form DNA formed in an alternating purine-pyrimidine sequence. The purine nucleobases are in anti-conformation whilst the pyrimidines in syn-conformation, creating the characteristic zigzag backbone

1.3 DNA processes

1.3.1 DNA replication

The genetic information of an organism is stored in its DNA sequence, therefore, all organisms must duplicate their DNA without any errors prior to any cell division, ensuring the two daughter cells contain the same genetic code as the original cell.¹⁸ That process is called DNA replication, where the double-stranded DNA molecule is used as a template to produce two identical molecules.¹⁸ This is a semi-conservative

process, as each newly formed DNA molecule consists of one of the original strands, and is used as a template to synthesise its complementary second strand.^{19,20} DNA replication is initiated by the unwinding of the double helix by DNA helicases, breaking the hydrogen bonds between the base pairs, creating a Y-shaped replication fork. DNA polymerases are the key enzymes in the replication process, discovered in 1956^{21,22}, as they are responsible for the recruitment and correct placement of the nucleotides, synthesising the second strand (**Figure 1.4**). The double helix is made up of two antiparallel strands, therefore the two new strands must be synthesised in opposite directions, dictated by the 5' to 3' direction of their templates. DNA polymerases are unable to initiate replication, only add nucleotides to the 3' terminus of a chain. DNA primase synthesises short RNA primers at the initiation sites, allowing DNA polymerases to extend the primer and synthesise the complementary strand. DNA polymerases can only build in a 5' to 3' direction, allowing for continuous replication only for one strand, as the enzyme can build in the direction of the unwinding. This is called the leading strand. The second is copied discontinuously, as new sections are unveiled by the unwinding, having to return and copy them as they become available, called the lagging strand. This results in stretches of copied fragments, known as Okazaki fragments (**Figure 1.4**).²³ The RNA primers are removed by 5' to 3' exonuclease, allowing DNA polymerase to extend the fragments, and DNA ligases to link the final sections together.

Any mistake during the DNA replication process can have a significant impact on the cell. If left unresolved it can lead to mutations, passed on continuously through every subsequent cell division.¹⁸ Therefore, the fidelity of DNA replication allows approximately one mistake every 10^9 nucleotides copied, with specific mechanisms in place to fix any mismatch that may occur. DNA polymerases are self-correcting enzymes, constantly proofreading the new DNA strands. In the case of a mispairing, the enzyme can identify that whilst a phosphodiester bond may have been formed between the last well-placed nucleotide and the wrongfully added one, it is unable to create a base pair with the template strand. Upon identification, the 3' to 5' proofreading exonuclease clips off any unpaired nucleotides, continuing until enough residues have been removed to regenerate a base-paired 3' -OH site, allowing the continuation of DNA synthesis. DNA replication is one of the most important DNA processes, as it ensures the accurate conservation of the genetic information of an organism.

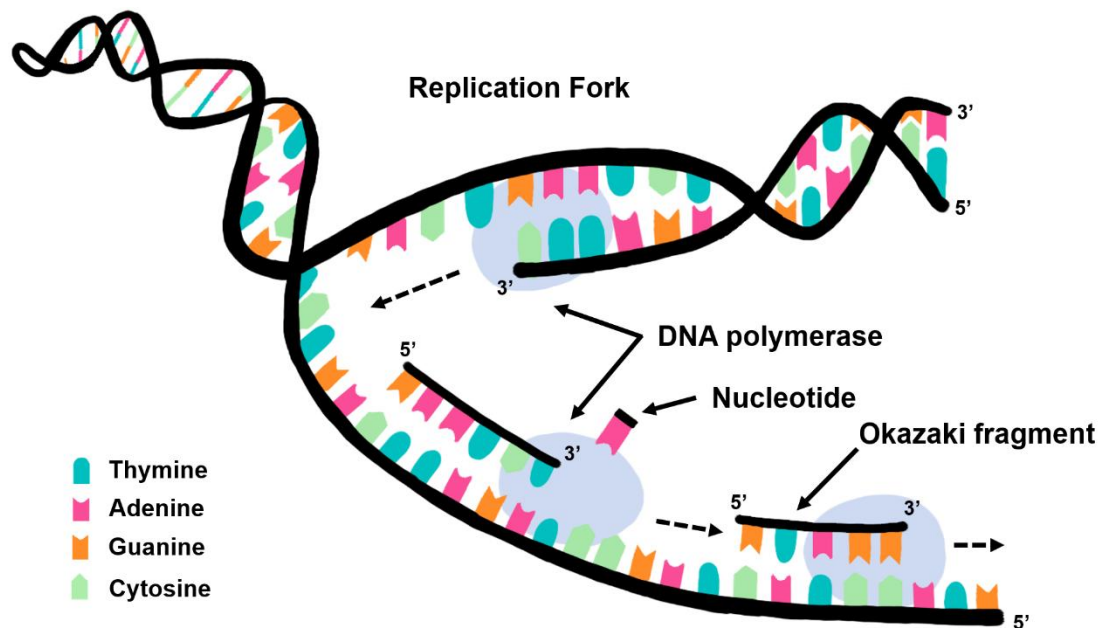


Figure 1.4: A diagram depicting DNA replication

1.3.2 DNA transcription

The expression of protein-coding genes is made up of two main processes, DNA transcription and translation. Some of the first studies investigating the mechanism and regulation of DNA transcription were conducted on bacteria in the 1960s.^{24–26} During transcription, a specific portion of a DNA sequence is copied into an RNA sequence. Like DNA, RNA is a linear polymer comprised of nucleotides linked through phosphodiester bonds, chemically differing from DNA in the sugar composition and nucleobase repertoire. RNA contains a ribose sugar in the backbone of each nucleotide, instead of a deoxyribose, and whilst it contains bases adenine, cytosine and guanine, its fourth base is uracil (U) replacing thymine. The same complementary base-pairing rules apply as DNA, with U and A forming a base pair instead.

Transcription is initiated by the unwinding of the DNA double helix at the site of transcription, marked by promoter regions located at the start of every gene. RNA polymerases, assisted by transcription factors, bind to the promoter region and locally unwind the double helix (**Figure 1.5**). One of the two DNA strands is chosen as the template strand, as RNA polymerase places complementary ribonucleotides in a 5' to 3' direction, forming a DNA/RNA base-paired hybrid. The localised unwinding of the double helix causes supercoiling of the DNA, to limit this effect the RNA polymerase is swiftly released from the promoter region. Unlike DNA replication, the transcribed DNA sections are allowed to reform the double helix, breaking the base pair bonds and producing a single-stranded RNA molecule, called messenger RNA (mRNA).^{27,28} RNA polymerases do not need RNA primers to synthesise the complementary strand,

therefore the continuous transcription moving along the length of the DNA, freeing the promoter, allows for multiple mRNAs to be produced simultaneously from the same DNA sequence. The transcription of the gene continues until the RNA polymerase reaches a terminator signal. The mRNA is then released from the DNA, allowing the helix to reform, and the RNA polymerase returns to the promoter to initiate a new transcription process.

The produced mRNA molecule is called pre-mRNA and undergoes further processing before continuing to translation, in eukaryotic cells. In every gene, protein-coding sequences, called exons, are interrupted by non-coding stretches of DNA, called introns. The pre-mRNA undergoes RNA splicing²⁹, a complex mechanism that removes the introns with high accuracy, linking the remaining exons together, to form the final mRNA sequence. The presence of both the introns and exons allows for the various proteins to be produced from the same gene, splicing the pre-mRNAs in diverse ways to generate mRNAs that will be translated into different proteins.

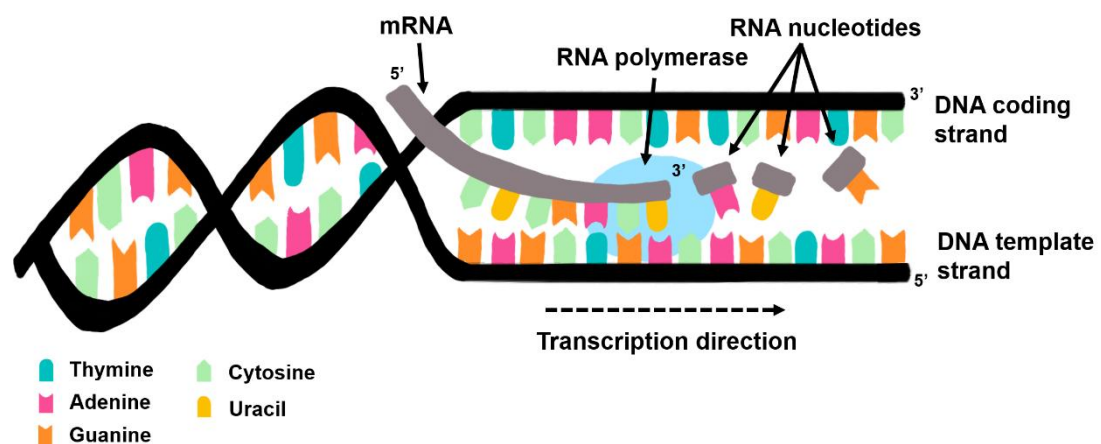


Figure 1.5: A diagram depicting DNA transcription

1.3.3 Translation

There are many types of RNA molecules, produced as the final product of certain genes.³⁰ However, most genes are transcribed into mRNA molecules, which undergo a process called translation, where the cell converts the genetic information into a protein molecule. Upon completion of the final mRNA, it travels from the nucleus to the cytoplasm where translation takes place. The mRNA sequence is read in consecutive groups of three nucleotides, called codons, each corresponding to an amino acid found in the genetic code. The four types of nucleotides can form 64 different triplet combinations, however there are only 20 amino acids.³¹ The deciphering of the genetic code occurred in the early 1960s, assigning all combinations to an amino acid, most associated with more than one trinucleotide

sequence.³² In addition, the trinucleotide AUG was discovered as the start codon, the translation of the mRNA begins upon identification of this sequence.³³ The translation process is catalysed by a RNA-protein complex called the ribosome, which threads the mRNA “decoding” the genetic information. Transfer RNA molecules (tRNAs) act as temporary carriers of amino acids, recognising and binding to a codon through its complementary sequence, an anti-codon, whilst carrying the corresponding amino acid. The protein is synthesised from N to C terminus, and the amino acids are bound to the tRNA molecule through an ester bond on the 3'-end of tRNA. When a tRNA is recruited and guided to the ribosome, the free amine of the bound amino acid attacks the carbonyl of the last amino acid added onto the polypeptide chain, releasing its tRNA (**Figure 1.6**). The cycle is continued, as the ribosome has three tRNA binding sites, displacing them upon release of their amino acid, moving along the mRNA chain. The end of the protein synthesis is signalled by the presence of one of three stop codons, UAA, UAG or UGA^{34–36}, not recognised by a tRNA, sending a signal to the ribosome to terminate the translation process. This process includes its own proofreading mechanisms, relying initially on the recognition of an incorrect fit by the ribosome. A secondary mechanism of kinetic proofreading identifies a wrongfully recruited tRNA due to a time delay that occurs in the event of an incorrect codon-anticodon pairing taking place. Finally, post-translational modifications occur upon completion of translation, incorporating non-RNA template-based changes to the chain, generating the final working protein.³⁷

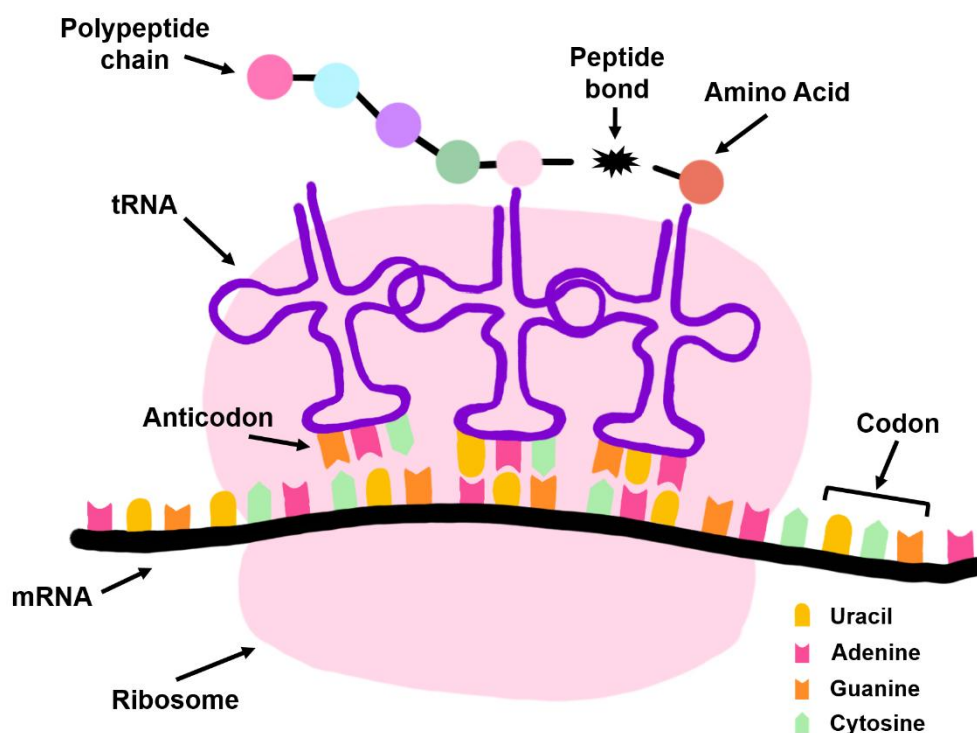


Figure 1.6: A diagram depicting translation

1.4 Secondary DNA structures

DNA can adopt a variety of alternative conformations based on sequence motifs and interactions with specific proteins, often referred to as non-B-DNA structures. This is an umbrella term for any forms of DNA that deviate from the B-form.³⁸ The seven most studied structures in chemical biology are the A-form double helix (A-DNA), four-way junctions (C-DNA), G-quadruplexes (G-DNA), triplexes (H-DNA), i-motifs (I-DNA), R-loops (R-DNA), three-way junctions (S-DNA) and the Z-form double helix (Z-DNA). **Figure 1.7** presents the structures of these alternative DNA complexes in alphabetical order, based on their one-letter codes.³⁹

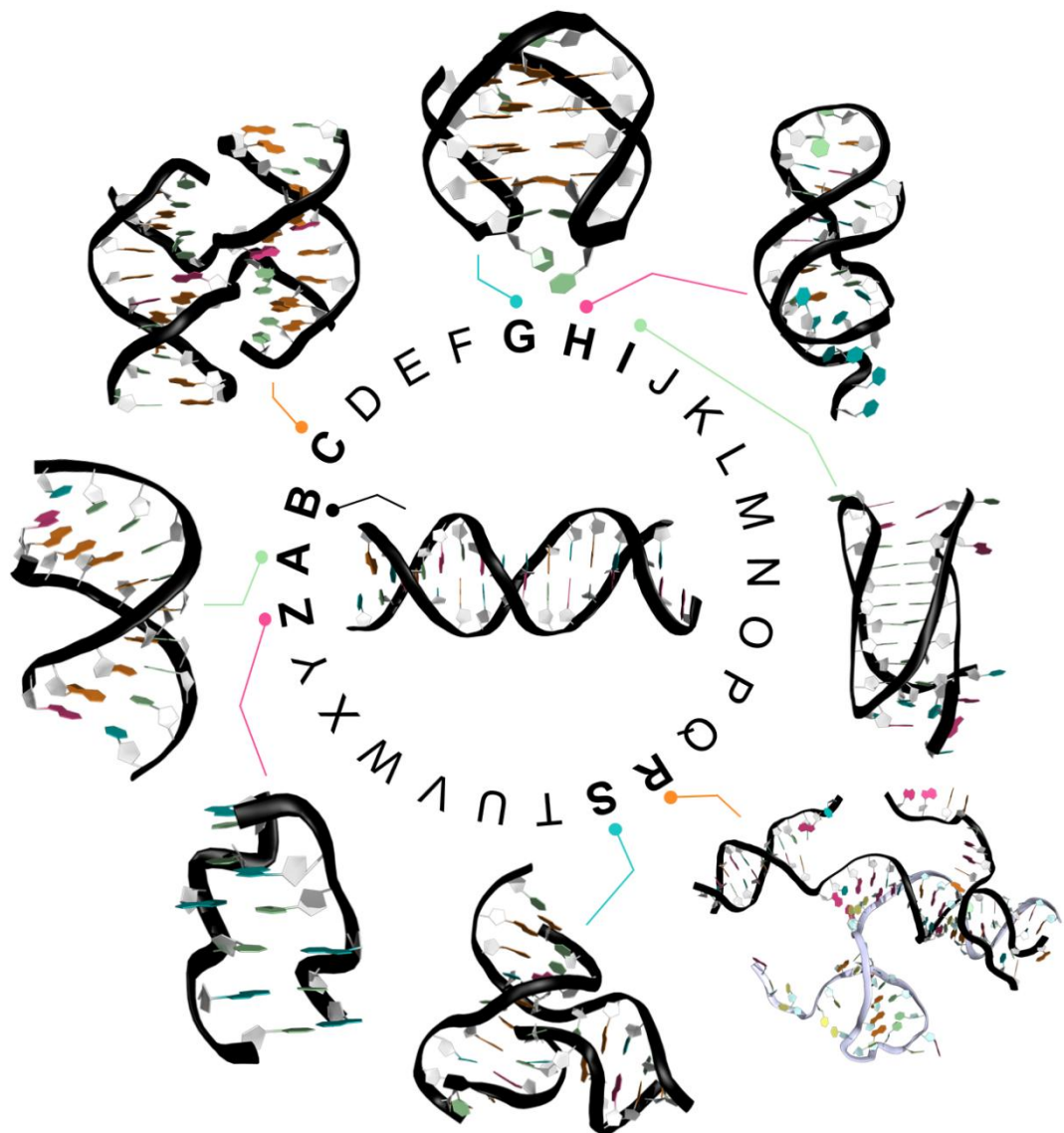


Figure 1.7: The alphabet of alternative DNA structures, A-form DNA (A-DNA, PDB **440D**), the classic B-form DNA (B-DNA, PDB **8YMZ**), four-way junctions (C-DNA, PDB **467D**), G-quadruplexes (G-DNA, PDB **9UK8**), triplexes (H-DNA, PDB **1B4Y**), i-motifs (I-DNA, PDB **8AYG**), R-loops (R-DNA, PDB **7D2L**), three-way junctions (S-DNA, PDB **1EKW**) and Z-form DNA (Z-DNA, PDB **4R15**).

Four-way junctions can fold from inverted repeats sequences⁴⁰, assembling cruciforms, therefore referred to as C-DNA. G-quadruplexes fold in G-rich sequences⁴¹, whilst i-motifs form in C-rich sequences.⁴² Triplexes can form from mirror repeats and three-way junctions in direct repeats⁴³, described as S-DNA as they are also known as slipped-DNA. Extensive research into the role and purpose of these structures has been carried out, associating alternative DNA structures with a plethora of cellular processes, leading scientists to investigate ways to control the folding and unfolding of these structures.

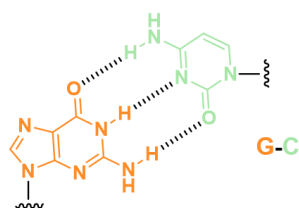
Here, the focus will be kept on four-way junctions, G-quadruplexes, i-motifs and three-way junctions, as these are the main non-canonical DNA structures that have been targeted with small molecules.

1.4.1 G-Quadruplex

1.4.1.1 Discovery and structure

In 1910, Bang reported that guanosine monophosphate (GMP) forms a gel at high concentrations, a property no other nucleotide possessed.⁴⁴ This discovery led to the elucidation that G-rich sequences may form high order structures. Almost 50 years later, using X-ray diffraction, Gellert *et al.* demonstrated that GMPs can assemble into a tetrameric structure, known as a G-quadruplex.⁴¹ The core of these structures consists of stacked G-quartets, described as square planar arrangements of four guanine bases held together through Hoogsteen hydrogen bonded base pairing (Figure 1.8).

Watson-Crick base pairing



Hoogsteen base pairing

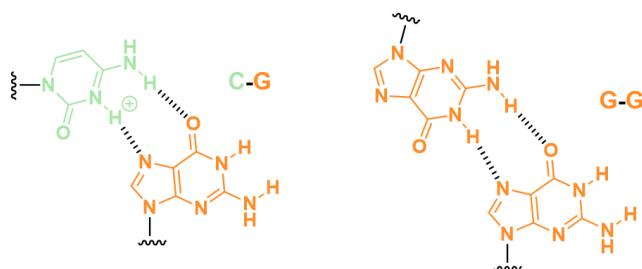


Figure 1.8: Watson-Crick and Hoogsteen base pairing between C and G

A G-quadruplex structure is comprised of stacked G-quartets with intervening sequences forming single-stranded loops between them, presented in **Figure 1.9**. This structure is further stabilised by monovalent cations occupying the central cavity, forming ionic interactions with the O₆ lone-pair electrons of each guanine, as well as π - π stacking interactions between the tetrads. Although all G-quadruplexes are similar to each other in principle, they can adopt many topologies, as a result of various possible combinations of strand direction, sequence composition and loop size.⁴⁵ These characteristics alongside sequence length, the number of tetrads and the binding cations all affect the topology and stability of a G-quadruplex.⁴⁶ Extensive research has been carried out to identify the biological relevance of G-quadruplexes. They have been associated with several cellular functions, including telomere biology and DNA damage, gene transcription and translation, mechanisms controlling genome stability and chromatin architecture.^{47–50} The varied involvement of G-quadruplexes made them an attractive target, leading to the design of a plethora of small molecules targeting them.^{51–53}

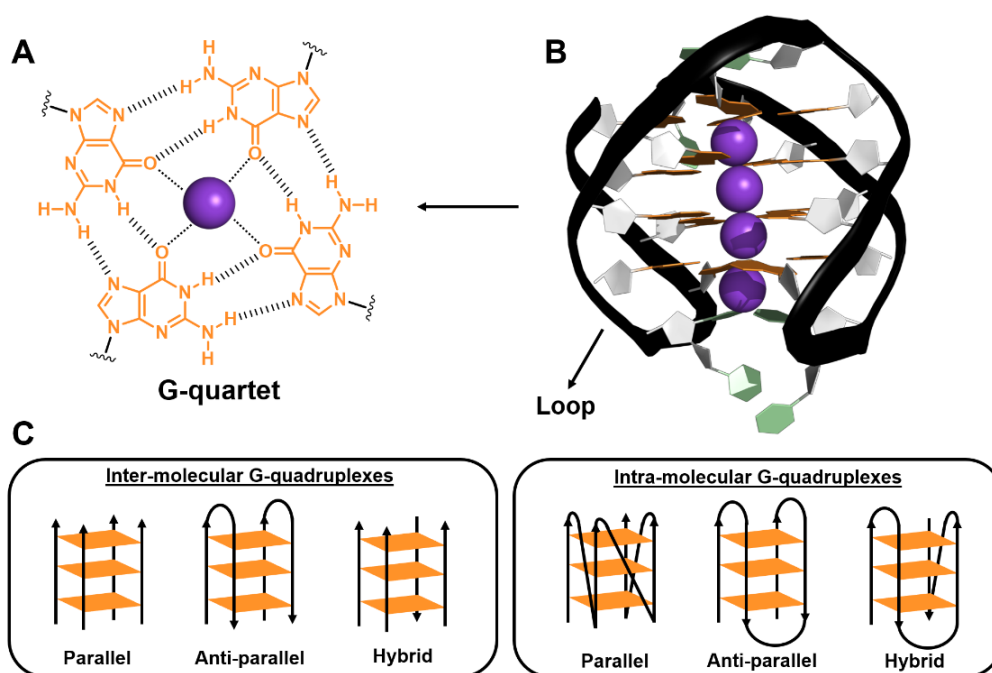


Figure 1.9: **A.** G-quartet assembled by four guanine bases interacting through hydrogen bonds, **B.** Crystal structure (PDB 9UK8) of an anti-parallel G-quadruplex, stabilised by potassium ions, **C.** schematic representation of possible inter- and intra-molecular G-quadruplexes in parallel, anti-parallel and hybrid topologies.

As an example, telomeres are regions of single-stranded DNA made up of 5'-TTAGGG repeats, located at the end of chromosomes, able to readily assemble into G-quadruplexes. Up to four consecutive G4 structures can form in these sequences, confirmed via X-ray crystallography⁵⁴ and NMR studies.^{55–62} Their potential in therapeutic use was first proposed in 1997, to target a reverse-transcriptase enzyme

called telomerase, expressed in up to 80% of cancer cells.^{63–65} Telomerase is responsible for the continuous elongation of telomeres, protecting the chromosomes from degradation whilst maintaining them in an immortal state.⁶⁶ As telomerase is inactive in healthy cells after early development, it is a good target for selectively targeting cancer cells. Inhibition of telomerase could be accomplished through stabilisation of G-quadruplexes in telomeric DNA, indirectly inhibiting the enzyme's activity and reversing cellular immortalisation. Several small molecules have been designed to target this process, identifying various G-quadruplex binders with anti-telomerase activity.^{53,67,68}

1.4.2 G-quadruplex binding compounds

1.4.2.1 Telomestatin

Telomestatin (**1.1**) is a natural product, isolated in 2001 by Shin-ya's group⁶⁹, from the species *Streptomyces annulatus* 3533-SV4, well studied as one of the most potent natural product inhibitors of telomerase^{69–84}. It is a cyclic compound made up of eight aromatic rings (**Figure 1.10**), its planar nature allowing for good overlap with G4 tetrads. Extensive biophysical and biological studies have been carried out, supporting that telomestatin can induce and significantly stabilise G4 structures ($\Delta T_m = 24\text{ }^{\circ}\text{C}$, $1\text{ }\mu\text{M}$).⁸¹ Additionally, a telomerase repeat amplification protocol (TRAP), used to detect reverse-transcriptase activity of telomerase⁸⁵, showed strong inhibition with an $\text{IC}_{50} = 5\text{ nM}$ ⁸⁶, however some discrepancies in this value have been uncovered in subsequent studies.⁸⁷ Promising anticancer activity was measured, causing telomeric shortening and apoptotic death in myeloma⁸⁸ and leukaemia cell lines.^{89,90} Due to the complexity of synthesising telomestatin^{91,92}, similar macrocyclic polyoxazoles have been developed.^{93–96} It is described as a selective G4 binder, displaying a 70-fold selectivity for G-quadruplexes over dsDNA.⁷⁶

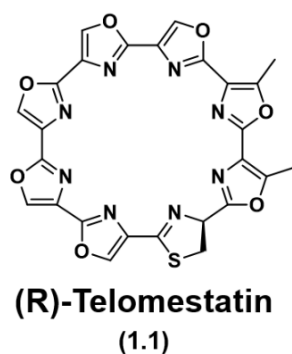


Figure 1.10: *R* enantiomer of telomestatin chemical structure (**1.1**)

1.4.2.3 TMPyP4

One of the first and most thoroughly studied synthetic small molecules, in the field of G-quadruplex stabilisers, is TMPyP4 (**1.2**).^{97,98} It is a tetracationic porphyrin structure (**Figure 1.11A**) and has been associated with RecQ helicase inhibition, telomerase inhibition and MYC downregulation.^{99,100} Anticancer activity has been measured in several cancer cell lines, including leukaemia and retinoblastoma cells, through telomerase inhibition and telomere-induced DNA damage.^{88,101–105} However, another study investigating the ligand's activity suggested that it could have cytotoxic effects on normal tissues.¹⁰⁶ **1.2** greatly increases G4 stability upon binding ($\Delta T_m = 17\text{ }^{\circ}\text{C}$, 1 μM), with an $\text{IC}_{50} = 6\text{ }\mu\text{M}$ for telomerase inhibition, measured through a TRAP assay.^{107–109} One of the major drawbacks of TMPyP4 is its lack of selectivity for G-quadruplexes, it binds well to DNA however displays no selectivity towards any structure of DNA.^{107–111} Further studies focused on TMPyP4 as a scaffold, altering the structure to improve selectivity¹¹², it has also been used as a “universal” DNA binding compound in assays.⁹² A pentacationic manganese(III) porphyrin (**1.3**) was presented by Dixon *et al.* (**Figure 1.11C**)¹¹², describing a DNA binder with a 10,000 fold selectivity for G-quadruplexes over duplex DNA. The structure of TMPyP4 makes up the core of the ligand, with the same extension added onto each arm, providing the selectivity. The compound inhibits telomerase with an IC_{50} of 580 nM.

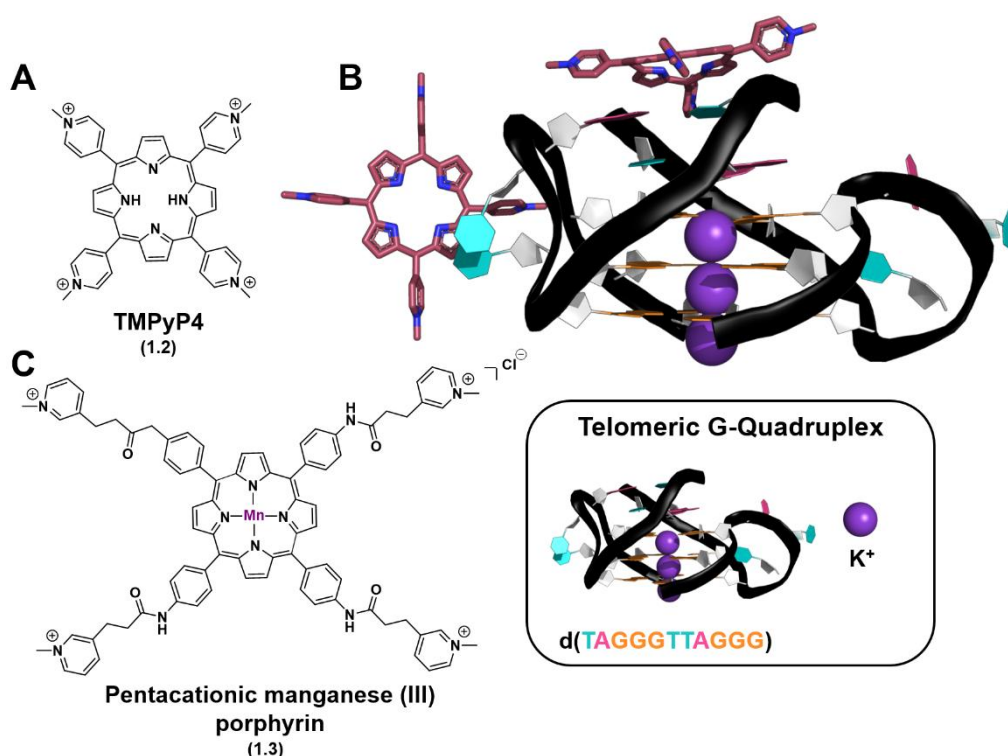


Figure 1.11: A. Chemical structure of TMPyP4, B. crystal structure of two TMPyP4 molecules bound to telomeric G-quadruplex DNA, C. TMPyP4 derivative, pentacationic manganese (III) porphyrin with 10,000 fold selectivity for G-quadruplex over duplex DNA.

1.4.2.3 BRACO-19

BRACO-19 (**1.4**) is a 3,6,9 - trisubstituted acridine derivative (**Figure 1.12B**), designed by Neidle and his group in 2001, through extensive computer modelling and crystallographic studies, studying trisubstituted acridines with G4 binding potential.^{68,113} *In vitro* assay results showed great promise for this compound, with a binding constant of 62.5 nM to human telomeric G4, demonstrated through SPR.¹¹³ A FRET-based melting assay revealed a G4 stabilising effect of 27 °C, and potent telomerase inhibition was measured (IC_{50} = 1,115 nM, TRAP assay), with an IC_{50} of 0.06 μ M, in human ovarian carcinoma cells.^{51,113} Compound **1.4** displays good selectivity for G-quadruplexes over dsDNA, calculated approximately a 31-fold preference.⁶⁸ This ligand has been assessed in various *in vivo* cancer environments, showing promise as an inhibitor of cancer cell proliferation.^{114–118} However, the compound's potential is limited due to its instability in physiological media, small therapeutic window and low membrane permeability.^{119,120} When in physiological media **1.4** is broken down through the hydrolysis of the amide groups in position 3' and 6' and the deamination of the dimethyl amine group in position 4' of the phenyl ring. Overall, BRACO-19 is a well-designed G4 binding compound, which has been further modified to optimise its promising properties.^{121,122}

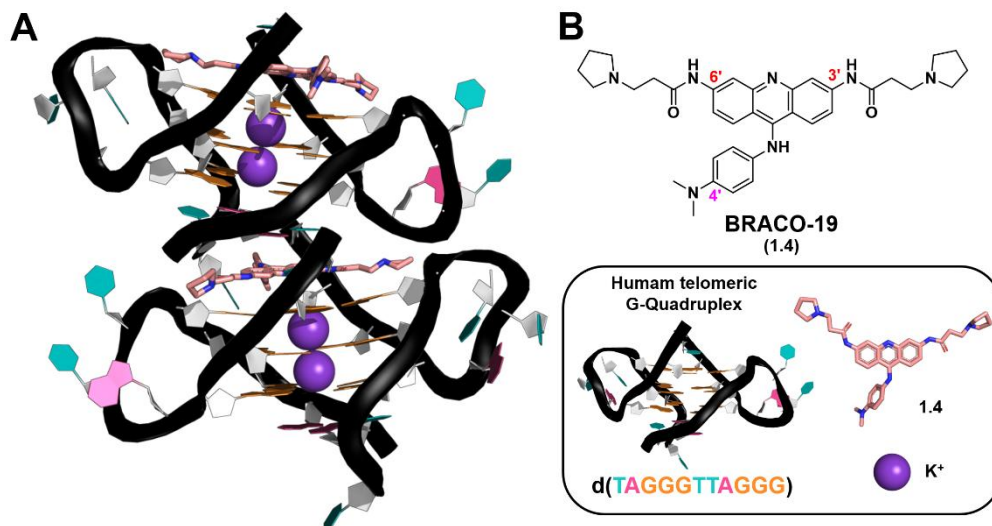


Figure 1.12: A. Crystal structure of a bimolecular parallel human telomeric G-quadruplex in complex with two BRACO19 molecules (PDB 3CE5), B. Chemical structure of BRACO19.

1.4.2.4 PhenDC3 (PhenDC6)

Another popular class of potent G-quadruplex ligands are phenanthrolines, known for their strong π - π stacking interactions with G-quartets.¹²³ PhenDC3 (**1.5**) and PhenDC6 (**1.6**) are closely related bisquinolinium compounds (**Figure 1.13A-B**). PhenDC3 has been associated with genetic instability through a series of

mechanisms in various species^{124–127}, whilst both ligands have been shown to stabilise RNA G4s that form in mRNA sequences, preventing translation.¹²⁸ PhenDC3 shows high selectivity for G-quadruplexes over dsDNA, directly assessed through a FRET-based melting competition assay, with a ΔT_m of 29 °C at 1 μ M, minimally affected in the presence of duplex DNA.¹²³ It can induce a conformation change from a hybrid to an antiparallel G4 structure, which shows potential for therapeutic use.¹²⁹ A fluorescence-induced displacement assay gave G4 binding affinities in the nanomolar range for both phenanthrolines.¹³⁰ Finally, a solution structure of PhenDC3 bound to the promoter G4 of *MYC* provides a good basis for further improvements to be investigated regarding this class of compounds.¹³¹ **Figure 1.13** presents PhenDC3 binding to G4s analysed through solution NMR, shown to bind via stacking on top of the G4, intercalating between the G-quartets or binding at the transition point in a duplex-G4 hybrid molecule.

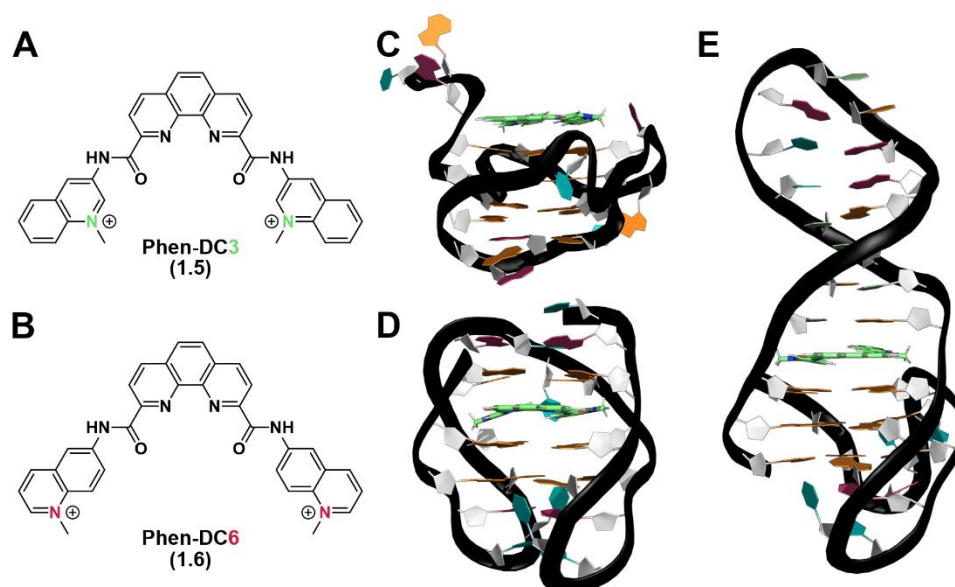


Figure 1.13: **A.** Chemical structure of Phen-DC3, **B.** Chemical structure of Phen-DC6, **C.** Solution structure of Phen-DC3 bound to a G-quadruplex, positioned on the top of the quadruplex (PDB 2MGN) **D.** Solution structure of Phen-DC3 intercalated into a human telomeric G-quadruplex, causing a hybrid to anti-parallel topology conformation (PDB 7Z9L) **E.** Solution structure of Phen-DC3 bound to a G-quadruplex-duplex DNA hybrid (PDB 8R6H). All three solutions structure were solved by solution NMR.

1.4.2.5 Pyridostatin

Pyridostatin (**1.7**) (PDS) is a G4 binding ligand (**Figure 1.14A**), disclosed by the Balasubramanian group in 2008, commercially available to purchase as a G4 stabilising agent.¹³² It selectively stabilises telomeric G4s, 35 - 44 °C (0.18 – 0.34 μ M) increase for G4s, minimally affected by the addition of dsDNA competitors, whilst only 0.5 – 1 °C increase for duplex DNA. A mechanical unfolding technique yielded a 490 nM binding constant to telomeric DNA. **1.7** was initially noticed for its effect on uncapping the POT1 protein from telomeres, however gained further attention for its

anticancer activity, inhibiting growth in osteosarcoma, fibrosarcoma and cervical cell lines, leading to senescence.¹³³ Like PhenDC3, **1.7** can cause structural alterations to G4 conformations upon binding. Due to the high affinity and selectivity of pyridostatin, it has been used as a biological tool to further study G4s. Some studies include visualising individual G4 formation in live cells¹³⁴, and mapping G4 distribution in the human genome, identifying 716,000 G-rich DNA sequences able to form G4s in the presence of PDS.^{135–137} The majority of G4s were non-randomly distributed in key regions, mainly telomeres, promoter regions of oncogenes and immunoglobulin switch regions.¹³⁸ The success of compound **1.7** has encouraged further research into designing improved analogues, with the help of structural studies breaking down the exact interactions that occur between the ligand and the G4s.¹³⁹

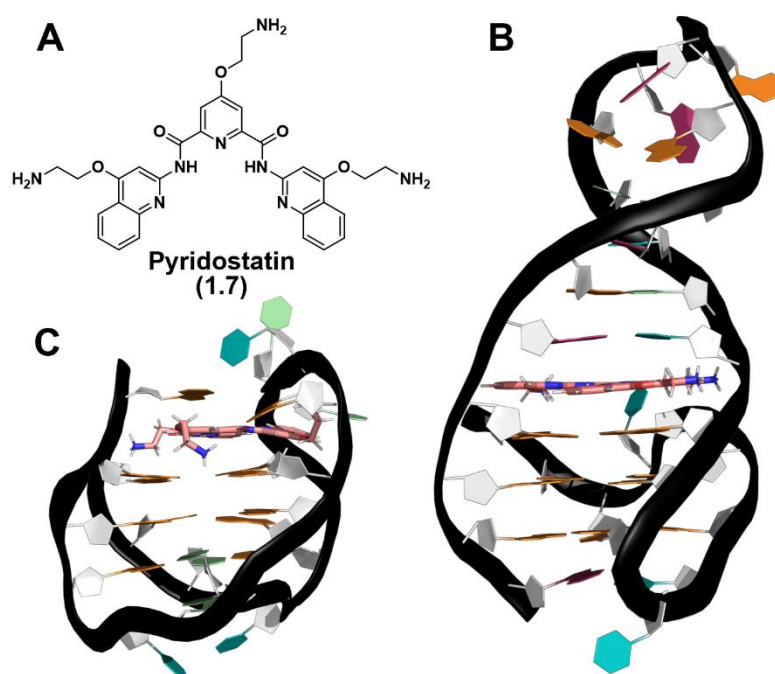


Figure 1.14: A. Chemical structure of pyridostatin, B. Solution structure of pyridostatin bound to a G-quadruplex-duplex DNA hybrid, C. Solution structure of pyridostatin bound to a G-quadruplex. Both solution structures were solved by solution NMR.

1.4.2.6 Quarfloxin (Pidnarulex)

Quarfloxin (CX-3543) (**Figure 1.15**) was the first G4 stabilising ligand to enter clinical trials.¹⁴⁰ It was rationally designed from norfloxacin, a quinolone antibiotic, and fluoroquinolone QQ5863¹⁴¹, a G4 ligand optimised by Cylene pharmaceuticals to eliminate its Topoisomerase II inhibition and maximise G4 selectivity. Quarfloxin was carried through to phase II as an antitumour agent, however, was discontinued due to bioavailability issues. Studies suggested the primary mechanism of action of the ligand targets rRNA synthesis, through displacement of nucleolin from G4s.¹⁴² Therefore, it was further optimised and a drug called Pidnarulex (CX-5461) was

designed to target RNA polymerase I (**Figure 1.15**).¹⁴³ It was later revealed that it is also a topoisomerase II poison.¹⁴⁴ Its nanomolar range binding affinity to G4, its high stabilisation and selectivity for G4s over duplex DNA, have led to its progression to a phase I oncology clinical trial.^{145,146}

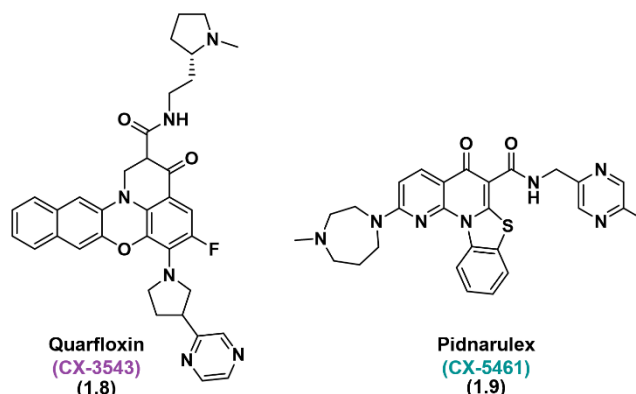


Figure 1.15: Chemical structures of quarfloxin (CX-3543) and pidnarulex (CX-5461)

1.4.2.7 QN-302

Naphthalene diimide derivatives have been extensively studied by the Neidle group as G4 stabilising ligands, investigated as anticancer agents against pancreatic cancer.^{147–150} The most promising candidate is a second generation tetrasubstituted naphthalene diimide known as QN-302 (**1.10**) (**Figure 1.16**).¹⁵¹ Its design was carefully carried out based on structure activity relationship studies of previous candidates.^{152,153} *In vitro* assays highlight 3 – 30 nM binding affinity to G4 structures and selective G4 stabilisation of a 10 – 18 °C (5 μ M) increase.¹⁵⁴ Single-digit nanomolar antiproliferative activity (1 – 6 nM) was exhibited when treating pancreatic cancer models with QN-302.¹⁵¹ The proposed mechanism of action describes strong binding and stabilisation of G4-forming sequences. **1.10** downregulates numerous cancer-related gene pathways and exhibits good bioavailability at therapeutic doses.^{151,154} This G4 ligand shows great therapeutic promise and is currently in clinical trials to treat solid tumours, including pancreatic cancer.¹⁵⁵

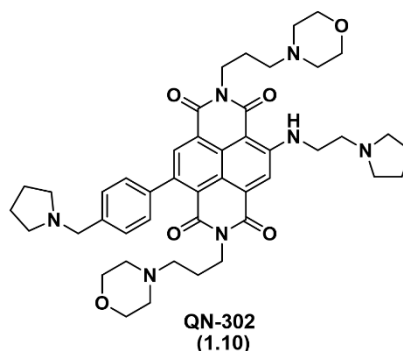


Figure 1.16: Chemical structures of QN-302 (1.10)

1.4.3 i-Motif

1.4.3.1 Discovery and structure

The first step toward the discovery of a different non-B-DNA canonical structure was taken when Marsh *et al.* solved a crystal structure of cytosine-5-acetic acid in 1962.¹⁵⁶ In 1993, Gehring *et al.* reported the first NMR resolved structure of a tetrameric DNA structure with protonated cytosine-cytosine base pairs, formed in the hexamer sequence d(5'-TCCCCC).⁴² Shortly after a complete characterisation of the intercalative quadruplex-helical tetramolecular structure was disclosed, named i-motif DNA (iM) (**Figure 1.17B**).^{157,158} They form in C-rich regions, made up of two parallel duplexes intercalated in an antiparallel conformation, held together by hemi-protonated cytosine-cytosine⁺ (C-C⁺) base pairs (**Figure 1.17A**).¹⁵⁸ The stability of the i-motif structure relies on the sequence length, number of consecutive cytosines, salt concentration, environmental pH, loop anatomy and temperature.^{159–166} The required protonation of the cytosine, to allow the hydrogen bond to occur, led to the belief that i-motifs could only form in acidic pH, limiting their biological relevance. However, this was contradicted through several studies that showed that iMs can form at physiological pH.^{167,168}

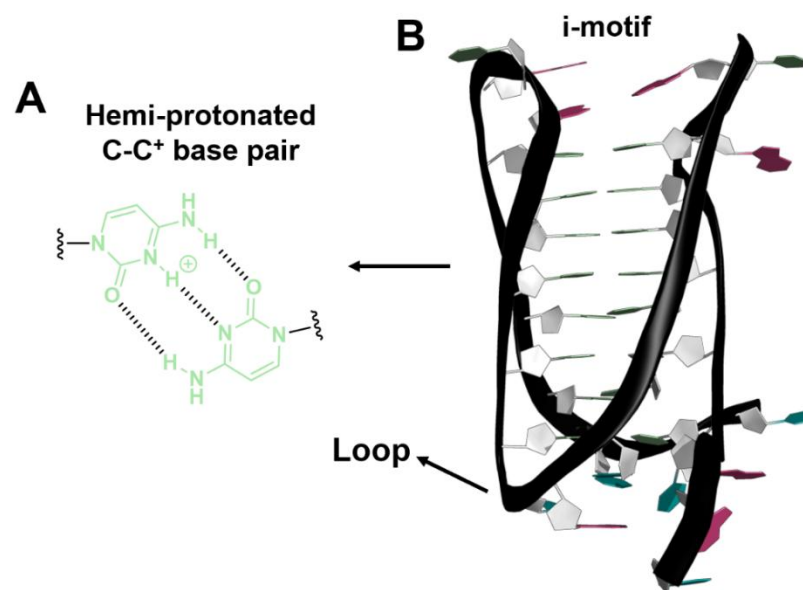


Figure 1.17: **A.** Base pair between hemi-protonated cytosine with cytosine through hydrogen bonds, **B.** Crystal structure of an intramolecular i-motif (PDB 8AYG)

Additionally, i-motifs have been identified and monitored in cells using iM-binding antibodies, in-cell NMR experiments, and more recently a whole genome mapping experiment.^{169–172} The latter entailed the first genome-wide mapping of i-motifs using an iMab (i-motif antibody)-based CUT&Tag workflow. i-Motifs are widely distributed in the genomes of many organisms including humans, plants and viruses, primarily

located in key processing regions, like telomers and gene promoters.^{171,173–178} Furthermore, they have been identified as key players in DNA replication inhibition, gene expression and telomerase activity modulation.^{179–185} The emerging evidence associating these DNA structures to critical functional processes have inspired the investigation of small molecules to bind and stabilise i-motifs.

1.4.4 i-Motif binding compounds

1.4.4.1 TMPyP4

The first small molecule studied able to bind i-motif structure was TMPyP4 (**Figure 1.18**) by Hurley *et al.* in 2000, previously discussed as a G4 binder.¹⁸⁶ UV titration experiments measured a 45 μM dissociation constant for a human telomeric i-motif, significantly lower than the values measured for a G4 (0.5 μM) and duplex DNA (1.2 μM).¹⁸⁷ No significant stabilising effect was measured when tested via a melting experiment. However, later studies measured a K_d of 1 μM for i-motif binding, suggesting the ligand had more than one binding mode.¹⁸⁸ Due to the promiscuous nature of TMPyP4, displaying no selectivity towards i-motif structures, limiting its reliability to studies working on pure isolated i-motif DNA. However, the structure acts as a good lead compound to develop more selective small molecules.

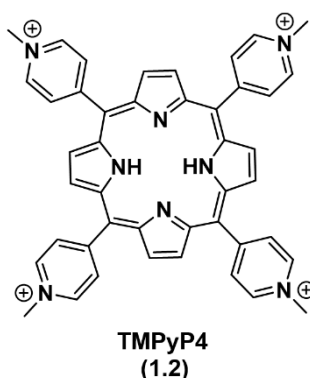


Figure 1.18: Chemical structure of TMPyP4 (1.2)

1.4.4.2 Mitoxantrone

A FRET-based DNA melting assay was used by Prof Waller and colleagues to screen a compound library from MicroSource, for any potential i-motif binding compounds, tested against a human telomeric i-motif sequence.¹⁸⁹ The screen highlighted mitoxantrone (**1.11**) (**Figure 1.19**) as a promising candidate, an anthraquinone derivative known as a topoisomerase II inhibitor, currently used in the clinic as an antitumour agent. SPR experiments were used to calculate K_d values of 12 μM for two i-motif structures (c-myc and hTeloC), compared to 71 μM for duplex DNA, indicating an i-motif preference. Additionally, mitoxantrone greatly stabilises i-motifs

under both acidic and physiological conditions ($\Delta T_m = 30 - 40\text{ }^{\circ}\text{C}$, $10\text{ }\mu\text{M}$), significantly higher than duplex DNA ($\Delta T_m = 6\text{ }^{\circ}\text{C}$, $10\text{ }\mu\text{M}$) and a G-quadruplex ($\Delta T_m = 16\text{ }^{\circ}\text{C}$, $10\text{ }\mu\text{M}$).¹⁸⁹ An approximate 5-fold preference for i-motif structures was indicated over dsDNA. Further optimisation could potentially increase selectivity.

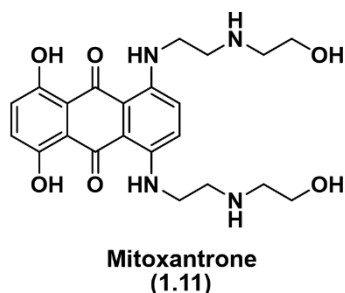


Figure 1.19: Chemical structure of mitoxantrone (1.11)

1.4.4.3 IMC-48 and IMC-76

An iM forming sequence in the promoter region of *bcl-2* was found to exist between a dynamic equilibrium of an i-motif and a hairpin structure. A FRET high throughput assay was used to screen a series of compounds against the sequence, detecting any compounds able to shift the equilibrium by stabilising the i-motif structure.¹⁹⁰ Compounds IMC-48 (1.12) and IMC-76 (1.13) (**Figure 1.20**) were identified having opposite mechanisms of action, stabilising the i-motif and the hairpin structures respectively. A dissociation constant of $0.49\text{ }\mu\text{M}$ was measured for IMC-48, indicating strong binding, although no stabilisation of the structure was observed by circular dichroism (CD) melting experiments. The biological effects of IMC-48 were studied in lymphoma cell lines, showing transcriptional activation and increased gene expression. This study provided the first definitive evidence that stabilisation of an i-motif structure can regulate gene expression. Sequence selectivity was detected as no change was observed in the gene expression of c-MYC and VEGF, both containing - i-motif in their promoter regions.^{191,192}

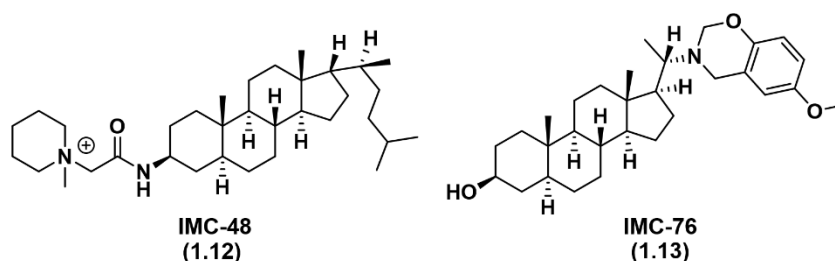


Figure 1.20: Chemical structures of IMC-48 (1.12) and IMC-76 (1.13)

1.4.4.4 Single-walled carbon nanotubes

Single-walled carbon nanotubes (SWNTs) are nearly one-dimensional structures made up of a single long graphene sheet, forming a nanotube.¹⁹³ In 2006, a study by

Li *et al.*, discovered that modified SWNTs can inhibit duplex DNA, inducing the formation of i-motif DNA under physiological conditions.¹⁹⁴ Melting experiments indicated high stabilisation of telomeric i-motif ($\Delta T_m = 22\text{ }^{\circ}\text{C}$, $10\text{ }\mu\text{g}\cdot\text{mL}^{-1}$), with no effect on G-quadruplex DNA, suggesting the development of the first selective i-motif binder, unable to bind to G4s. SWNTs can inhibit telomerase activity through stabilisation of i-motif structure, assessed via a TRAP assay.¹⁸⁵ These promising results led to the investigation of graphene nanoparticles, namely modified graphene quantum dots (GQDs). GQDs were studied as i-motif binders with the telomere and cMYC sequences, displaying high stabilisation of the i-motif structures, with ΔT_m values of $20\text{ }^{\circ}\text{C}$ and $16\text{ }^{\circ}\text{C}$ at $20\text{ }\mu\text{g mL}^{-1}$, respectively.¹⁹⁵ They can also induce the formation of i-motifs at physiological pH, even up to pH 8.0.

1.4.4.5 Compound NSC202386

In 2025, Prof Waller and colleagues characterised a new i-motif forming sequence, located in the promoter region of the Nrf2 gene.¹⁸⁰ A fluorescent indicator displacement (FID) assay was carried out, screening 1584 drugs for potential i-motif binding properties. A total of 11 compounds were identified, with compound NSC202386 (**1.14**) (**Figure 1.21**) exhibiting the most promise. Upon treatment with compound **1.14**, Nrf2 expression was significantly decreased. However, melting experiments of a C-rich Nrf2 sequence suggested a destabilising effect by the ligand. UV titration experiments were performed to determine dissociation constants (K_{ds}), measuring 250 nM for the i-motif structure, a 2-fold preference over the G4 and a 7-fold preference over duplex DNA. Finally, a 6-fold sequence selectivity of **1.14** for the Nrf2 i-motif was measured, over other i-motif forming sequences.¹⁸⁰

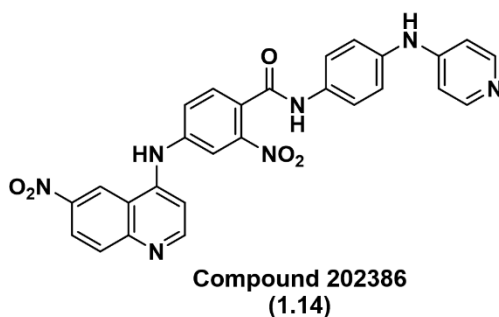


Figure 1.21: Chemical structure of compound 202386

1.4.4.6 Ellipticine

Ellipticine (**1.15**) (**Figure 1.22**) is a plant alkaloid, a polycyclic molecule with antitumour activity.^{196,197} A recent study in 2020, demonstrated its i-motif binding abilities, highly stabilising the i-motif structure ($\Delta T_m = 20 - 30\text{ }^{\circ}\text{C}$, $50\text{ }\mu\text{M}$) over G4 and

duplex DNA ($\Delta T_m = 2 - 3\text{ }^{\circ}\text{C}$, $50\text{ }\mu\text{M}$) in a FRET-melting experiment.¹⁹⁸ Further circular dichroism experiments supported this i-motif selectivity. Both assays were repeated at physiological pH, verifying ellipticine can induce and stabilise the i-motif structure at neutral pH. Ellipticine has previously been shown to intercalate into duplex DNA, therefore would need further optimisation to enhance its i-motif binding selectivity.¹⁹⁹

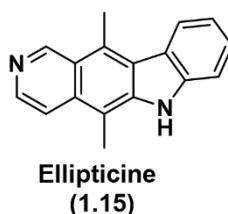


Figure 1.22: Chemical structure of ellipticine (1.15)

1.4.5 Three-way junctions

1.4.5.1 Discovery and structure

Three-way junctions, also known as slipped-strand DNA (S-DNA), were first described in 1964 by Kornberg *et al.*, as intermediates of enzymatic DNA synthesis.^{200,201} A 3WJ is an alternative DNA structure that consists of three duplex DNA sections connected at a single point, the branch point, forming a Y-shaped structure (**Figure 1.23A**). Initial studies focused on the visualisation of branched DNA in the DNA of T4²⁰² and T7²⁰⁰ bacteriophages, *Pneumococcus*²⁰³ and *Xenopus laevis* oocytes.²⁰⁴ However, the precise structure of the 3WJ was not fully understood until Lilley²⁰⁵ and Kallenbach's²⁰⁶ groups proved that a fully matched 3WJ adopts a well-defined stable Y-shaped structure.

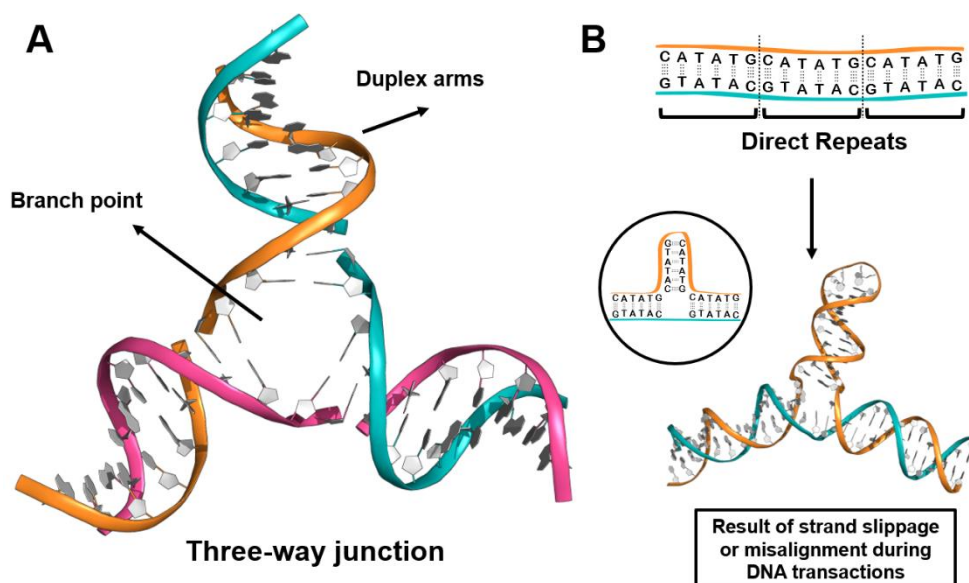


Figure 1.23: **A.** Crystal structure of a three-way junction (from PDB 1DRG), **B.** Schematic representation of a sequence made up of direct repeats, forming a three-way junction as a result of a strand slippage or misalignment during DNA transactions (structure was generated with alphafold3).

Gel and FRET experiments confirmed that in a perfectly base-paired 3WJ the distances between the duplex arms are almost equal, forming a pyramid shape structure. 3WJs can naturally form in the presence of direct repeats, described as stretches of DNA where a specific sequence is repeated multiple times in tandem (**Figure 1.23B**). They can form as intermediates during various cellular processes, including DNA replication, repair and recombination and have been associated with various diseases linked to direct repeat sequences.^{43,207–211} The recent publication of the complete sequence of a human genome, identified that 18.6% was made up of direct repeat sequences, highlighting the relevance of targeting three-way junctions as they can form in these regions.^{212,213}

1.4.5.2 Therapeutic value

In eukaryotic cells, the unwinding of the double helix during DNA replication causes torsional stress to the double-stranded sections ahead. As a result, S-DNA structures can transiently form, either including a single-stranded loop or a hairpin, shown to be topologically equivalent to unwound DNA, therefore favoured by negative DNA supercoiling.²¹⁴ Stabilisation of these structures creates a physical obstacle to the replication machinery, stalling or completely inhibiting the replication process.

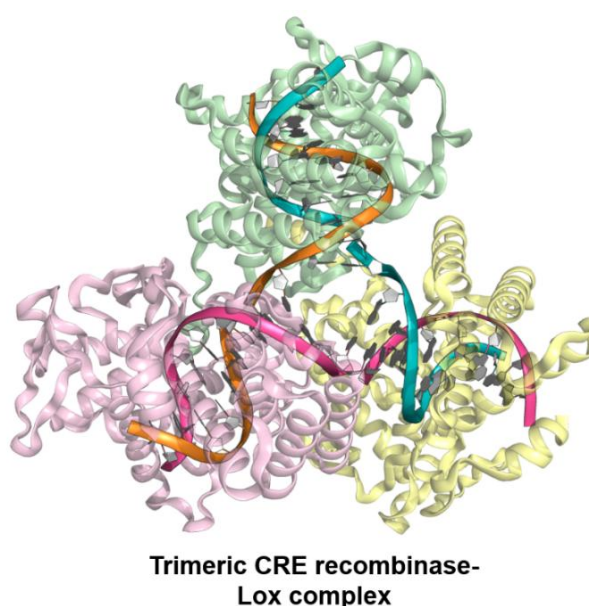


Figure 1.24: Crystal structure (PDB 1DRG) of a 3WJ with a trimeric Cre recombinase-Lox complex

Three-way junctions can form as recombination intermediates during the repair of double-stranded DNA breaks, with the first crystallographic representation of a 3WJ published in 2001, forming in a Cre-lox recombination system.²¹⁰ A novel complex was identified, outlining a Cre recombinase trimer bound to a Y-shaped 3WJ (**Figure 1.24**), mimicking previously characterised tetrameric complexes.

Additionally, three-way junctions have also been heavily studied in association with the expansion of 13 trinucleotide repeats, linked to over 40 degenerative human disorders, including Huntington's disease and myotonic dystrophy.^{209,215–221} Normal repeat tracts extend from five to twenty four repeats, however in disease-associated expansions the length varies from one hundred up to a thousand repeats, displaying great genetic instability.⁴³ The most extensively studied repeat sequence is (CTG)(CAG), responsible for sixteen types of diseases. Studying these sequences will shed light onto the role of the 3WJ intermediate, and its effects on the genetic instability associated with the expansion repeats. 3WJs can form during local misalignment of DNA during replication and repair, with the slipped strand forming a hairpin or an unpaired loop. Whilst no expandable repeats can form perfect hairpins, they can form imperfect hairpins containing mismatches.^{218,222–228} The mismatches prevent spontaneous 3WJ formation in a double helix, however their formation can occur following a DNA unwinding event, such as DNA replication, repair or transcription, followed by the self-annealing of the slipped strand to form the hairpin. Whilst little is known regarding the role of 3WJs in repeat sequences, a study on the (CTG)(CAG) sequence, demonstrated that 1 to 10 repeat-long slipped DNA structures can form anywhere along the tract, forming both hairpins and unpaired loops in the CTG and CAG sequences respectively.⁴³

Three-way junctions are one of the lesser studied alternative DNA structures, however, show great promise in developing new therapeutic tools. Additionally, the design of 3WJ-binding complexes would aid in uncovering the extent of their involvement in the various cellular processes discussed. A complete review of the reported 3WJ-binding compounds to date is presented in **Chapter 3**.

1.4.6 Four-way DNA junctions (Holliday Junction)

1.4.6.1 Discovery and structure

The structure of a four-way junction was first described by Robin Holliday in 1964, that is also known as a Holliday junction.²²⁹ It is an intermediate structure in homologous recombination (HR), that can exist in two conformations, open and X-stacked (**Figure 1.25**).^{230,231} HR is a genetic rearrangement pathway that occurs between two identical strands of DNA that come together and exchange genetic information. During this process, formation of a Holliday junction is essential for the process to be carried out equally and reciprocally from both DNA molecules. HR is a key cellular process that occurs in meiosis and DNA repair, repairing double-strand

breaks (DSB) in DNA, ensuring genetic integrity.⁵ DNA repair mechanisms, focusing on homologous recombination, will be discussed in detail in **Chapter 4**.

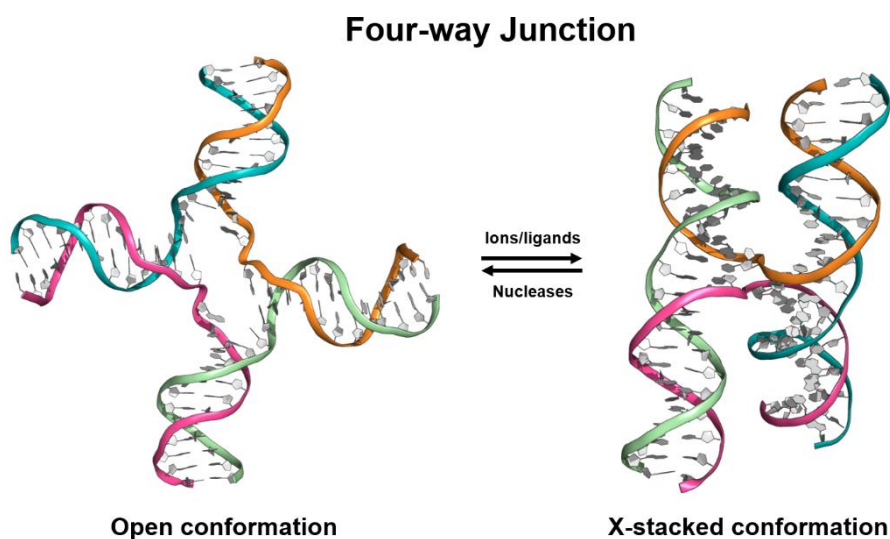


Figure 1.25: A four-way junction DNA structure in open (from PDB 3CRX) and X-stacked (from PDB 2WJ0) conformations

1.4.6.2 Structure Resolution

The structure of the four-way junction was proposed by Lilley and his group in 1988, through a series of gel electrophoresis experiments.²³² Four 80 bp long sequences were carefully designed, that assemble into a single 4WJ structure. Each duplex arm of the junction was 40 bp in length, each containing a unique restriction site approximately 12 bp from the centre of the junction (**Figure 1.26**). Restriction sites are short DNA sequences selectively recognised by restriction enzymes that cleave DNA at this site. Therefore, through the addition of the selected enzymes, specific arms of the junction could be cleaved, shortening the arms by 25 bp each, having no effect on the rest of the structure.

The 4WJ was subjected to pairwise cleavage of the arms by addition of the enzymes, resulting in six enzyme pairs. All six cleaved junctions were analysed by gel electrophoresis alongside the full 4WJ structure, in the presence of 5 mM Mg^{2+} in the running buffer.²³³ The full junction migrated the slowest down the gel due to its size as a clean narrow band, confirming a single structure, whilst the cleaved junctions migrated faster, displaying two products formed for each combination. The most retarded band was verified to correspond to a three-arm structure, with only one shortened arm, and the second band that migrated faster corresponding to the two-arm structure. Whilst all the three-armed structure migrate at a similar rate, the two-armed structures migrate at three different rates, in pairs.

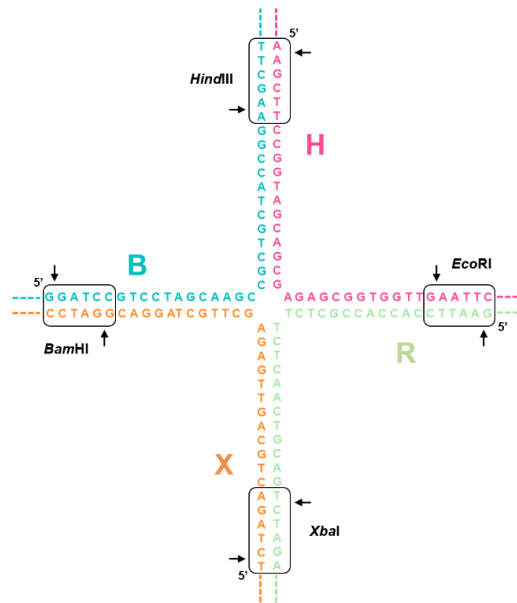


Figure 1.26: The central region of a four-way junction assembled by the hybridisation of four 80 nucleotide-long oligonucleotides (X, R, H, B) with highlighted restriction sites

Analysis of these results, suggested that an acute angle between the junction arms causes slower migration, agreeing with the principle that retardation increases with DNA curvature (**Figure 1.27A**). Three more sequences were assessed, changing only two base pairs from the original at a time. The base pairs altered in two (junctions 2 and 3) were located directly at the centre of the junction, altering which two-arm structures were moving at the different rates. The final sequence (junction 4) differed in the second set of base pairs from the centre, indicating similar results to the first structure. Lilley and colleagues concluded that the junction was able to undertake two X-stacked isomer forms, with overlapping helices, primarily determined by the base pair sequence at the branch point of the junction.

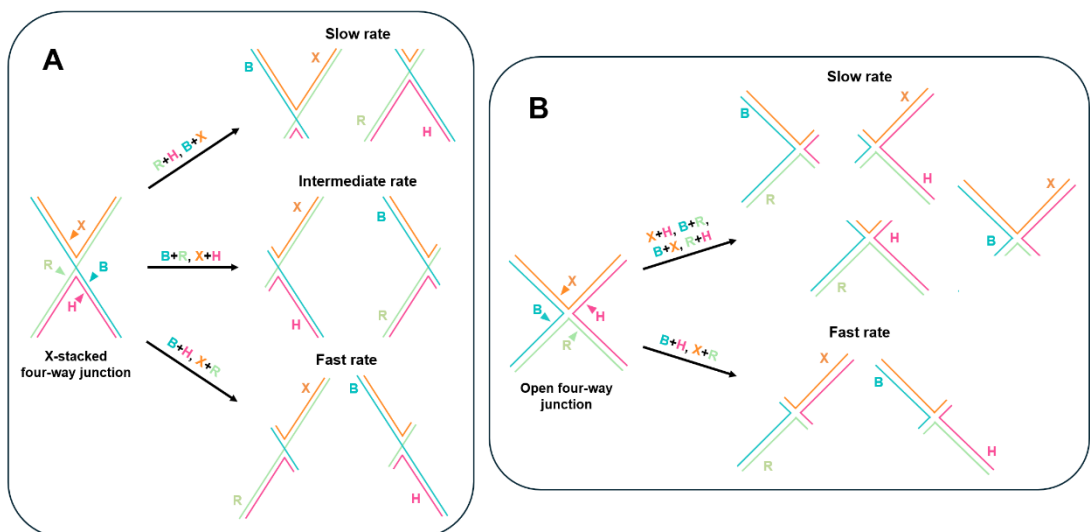


Figure 1.27: PAGE experiment results, junction species formed by restriction enzyme cleavage of a four-way junction in the **A.** presence of Mg^{2+} , **B.** absence of Mg^{2+}

To directly analyse the effect of the high salt environment on the conformation of the junction, the experiments for junctions 1 and 2 were repeated in the absence of Mg^{2+} (**Figure 1.27B**).²³² The gel results supported the model that the idea that the junctions were no longer in the X-stacked structure, with the two-arm structure products of both junctions behaving the same way, resembling a square-planar structure with a 4-fold symmetry. The proposed reasoning described that the phosphate repulsion is minimised upon coordination of ions, allowing the helix-helix stacking structure. However, in the absence of divalent ions the strength of the repulsion holds the four duplex arms as far as possible from each other. This set of PAGE experiments defined that four-way junctions can exist in two conformations. The open and X-stacked conformations are undertaken in low and high salt level environment accordingly.²³²

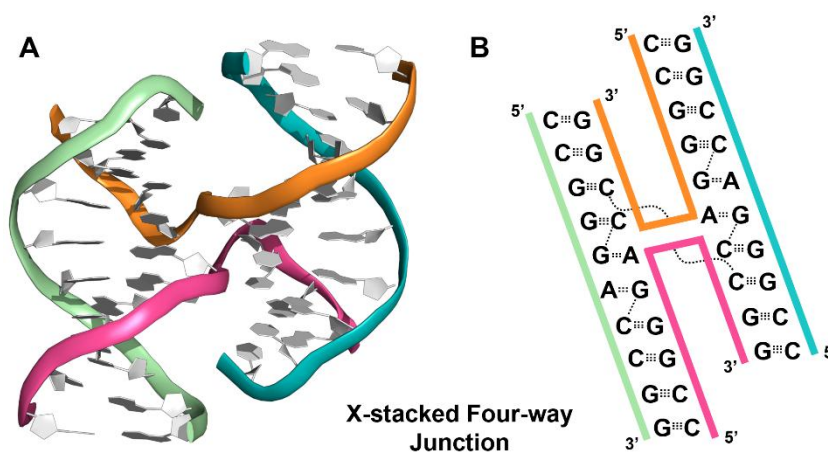


Figure 1.28: **A.** The first crystal structure of a four-way junction (PDB 467D), **B.** Diagram of the X-stacked crystal structure four-way junction

The first crystal structure of a four-way junction was obtained in 1999 by Coll and his group, confirming the structure proposed by Lilley through solution experiments (**Figure 1.28A**).²³⁴ The crystallised X-stacked conformation of a 4WJ was made up of two continuous B-DNA helices running antiparallel to each other, stacked at a 60° angle (**Figure 1.28B**).

1.4.6.3 Therapeutic value

The proposed Holliday Junction intermediate in HR was visualised in eukaryotic cells in 2010, proving its important role in this pathway.²³⁵ Extensive research has been carried out on understanding the exact consequences of HJ resolution in DNA damage repair and replication.^{236–239} The HJ intermediate is strictly regulated by various nucleases, ensuring its proper resolution, necessary for maintaining genome stability and cell survival, whilst improper HJ resolution has been associated with DNA replication disturbance, leading to chromosomal instability and mitotic collapse.^{240–242}

1.4.6.3.1 Alternative Lengthening of Telomeres (ALT) pathway

Additionally, the HJ has also been investigated as a target for the development of new cancer therapies. One pathway by which cancer cells accomplish immortalisation is telomere extension, briefly discussed in **section 1.4.1.1**. Telomeres are located at the end of the chromosome, protecting the genetic information from damage during cell division. With each cycle several bases are naturally lost, as DNA replication enzymes are unable to replicate the chromosome up to the last base. Thus, after multiple rounds of cell divisions, the telomeres have been progressively shortened, posing a limit to the number of times a cell can be replicated.^{243,244} Once the telomere sequences have been consumed, the DNA damage response mechanism is triggered to prevent the generation of an incomplete chromosome, inducing cell death due to replicative senescence.¹¹ Cancer cells take advantage of this pathway, reactivating telomerase, an enzyme used to create and elongate telomeres during early development. Up to 80% of cancer types rely on cell immortalisation by telomerase⁶⁴, however, most of the remaining 20% have developed a different mechanism, called the alternative lengthening of telomeres (ALT) pathway.^{65,245} The ALT pathway is a homology-mediated repair mechanism, which involves the formation of the HJ intermediate.²⁴⁶

A new therapy could be developed by targeting and stabilising this HJ, preventing its resolution, disrupting the ALT pathway and therefore the immortalisation of the cell. Due to the repeated sequence assembling the telomeres, a sequence-selective HJ-binding compound would make for a highly promising anticancer agent. Additionally, such a compound could be used as a diagnostic tool, identifying which cancer cells accomplish immortalisation through the ALT pathway.

1.4.6.3.2 DNA damage response (DDR) pathways

Genomic instability is a major consequence of the rapid proliferation of cancer cells, therefore the DNA damage response (DDR), a repair pathway, is recognised as a therapeutic opportunity to target cancer over healthy cells.²⁴⁷ DDR mechanisms are responsible for detecting and repairing DNA damage and are often defective in cancer cells. Many therapies used in the clinic take advantage of this characteristic, exploiting it with the addition of exogenous DNA damage and/or DDR inhibition.²⁴⁸

This therapeutic approach has been paired with synthetic lethality, a genetic interaction that occurs when two defected genes are tolerated by a cell individually, however when combined cause cell death.²⁴⁹ The most successful example of this combination therapy is described by the administration of PARP inhibitors (PARPi) to

BRCA1/2 deficient cancer cells. PARP enzymes are damage sensor and signalling molecules, and PARP1 is greatly involved in the detection of single strand DNA breaks.^{250,251} Targeting of PARP1 has been extensively studied as a cancer treatment, leading to the development of several PARP inhibitors.²⁵² Upon treatment of cancer cells with PARP1 inhibitors, increased sensitivity to DNA damaging agents was noted, proposing PARP inhibitors as chemosensitisers. Several studies have investigated the use of a combination approach, administering both a DNA damaging agent and a PARPi.^{253–255} This approach shows great promise as the sensitisation caused by the PARPi allows for a small dose of the cytotoxic DNA damaging agent to have the same effect.

A significant breakthrough was made in 2005, when two groups discovered that BRCA1/2-deficient cancer cells are considerably more sensitive to PARPis.^{256,257} The inhibition of PARP1 leads to the accumulation of single strand breaks (SSB), which turn into much more dangerous double strand breaks (DSB), when left unrepaired. PARPis have minimal effect on normal cells, as DSBs are repaired via homologous recombination, or by nonhomologous end joining (NHEJ). BRCA1/2 are involved in the regulation of HR, therefore BRCA1/2-deficient cells are defective in HR repair. The therapeutic approach is based on the administration of PARPi leading to an increase of DSBs, unable to be repaired via HR, leading to cell death. The NHEJ pathway is unable to repair all DSBs, especially ones that form in the replication fork, normally repaired by HR. This synthetic lethality approach led to approval of PARP inhibitors administered to cancer patients with BRCA1/2 mutations.^{258–261}

BRCA1/2 mutations are only associated with certain types of breast, ovarian, prostate and pancreatic cancer, limiting its application.^{262,263} This approach could be expanded as a treatment for more cancer types by selectively disabling the HR mechanism. As discussed in **section 1.4.6.3**, a four-way DNA junction (HJ) forms as an intermediate in this pathway. By stabilising the HJ and preventing its resolution, the HR pathways could be disabled in other cancer cells. Small molecules that selectively target the HJ, in combination with PARPis could be a new therapeutic tool in cancer treatment.

1.4.6.3.3 Inverted repeat sequences

In addition to their formation during homologous recombination, four-way junctions can readily form from inverted-repeat sequences (IRs). IRs are described as sections of DNA, made up of 6 or more nucleotides, where the second half of the sequence is reversibly complementary to the first. A recent sequencing of a whole genome disclosed that 1.6 % of autosomal DNA is covered by IRs, dispersed non-randomly in

the genome, noted in promoter regions and DNA replication initiation sites.²¹² Four-way junctions can form if the two complementary consecutive sequences form a hairpin in both strands of DNA, assembling a cruciform structure (**Figure 1.29**). The first crystal structure of a four-way junction in an inverted repeat sequence was reported in 2000 by Eichman *et al.*⁴⁰ Cruciform structures play functional roles in DNA replication, transcription, recombination and gene expression.^{264–266}

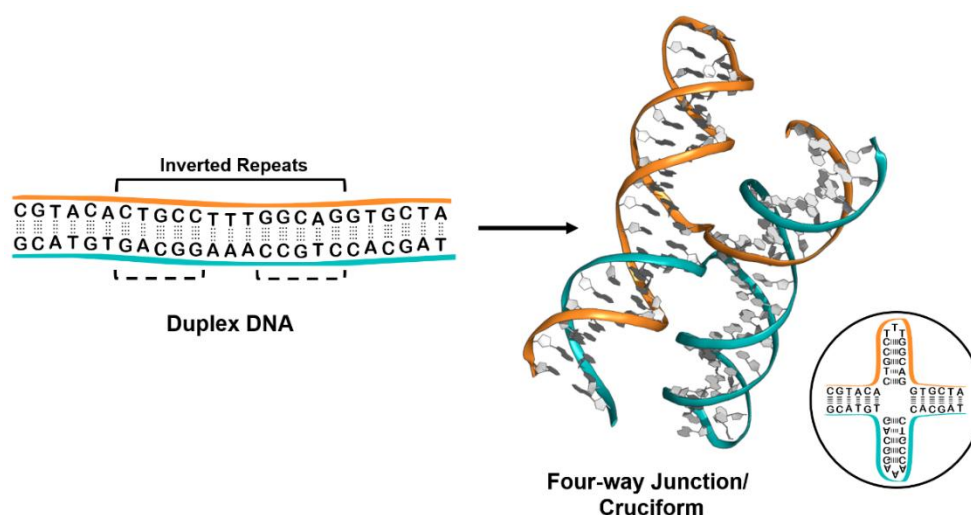


Figure 1.29: Inverted repeat sequences able to form a cruciform structure (structure generated with *alphafold3*)

IRs have been shown to cause genomic instability, through stimulating DSBs and chromosomal fragility in eukaryotic cells, causing chromosomal rearrangements.^{267–271} Cruciform structures that form in short IRs (<30 bp) stimulate DSBs by stalling DNA replication forks and/or activating enzymes that cleave them, causing deletions, leading to genetic instability.^{271,272} IRs have been associated with cancer aetiology, due to their increased presence at mutation hotspots in human cancer genomes, as well as their triggering of genetic instability, a hallmark of most cancer types.^{273–275}

Four-way DNA junctions are involved in several key biological processes of healthy cells, as well as associated with various diseases. Selective targeting of these alternative DNA structures could prove highly beneficial in the development of novel treatments, especially in cancer. A review of 4WJ-binding compounds reported to date is presented in **Chapter 2**.

1.5 Acridines as DNA junction binding compounds

1.5.1 Early studies

Acridines are a class of heterocyclic compounds with a planar aromatic surface, comprised of three fused six-membered rings. They were first described in 1870 by

Graebe and Caro as a type of impure anthracene extracted from coal tar, given its name due to its irritating nature on the skin.^{276,277} Their planar nature allows them to bind to DNA via intercalation.²⁷⁸

DNA intercalators are molecules that bind to DNA through reversible insertion between adjacent base pairs in double-stranded DNA, stabilising the helix. To accommodate the intercalator, the two base pairs are forced apart causing distortion in the DNA, as the helix partially unwinds and lengthens to allow for the insertion of the intercalator.^{279–281} The local deformation of the DNA backbone leads to structural and mechanical alterations to the chain, disrupting DNA replication and transcription, often leading to cell death. The structures typically consist of polyaromatic systems, and stabilisation occurs through a combination of π - π stacking, hydrophobic and electrostatic interactions, as well as hydrogen bonding and Van der Waals forces.

Amsacrine (**Figure 1.30**) is a 9-anilinoacridine derivative first reported in 1974 by Cain and Atwell, who were developing libraries of acridine derivatives, aiming to improve their antitumour activity.^{282,283} In 1978, amsacrine became the first synthetic DNA intercalating agent to enter the clinic, administered for the treatment of acute leukaemia and lymphomas. It is made up of the acridine base linked to a 4-amino-3-methoxymethane sulfonanilide via ring position 9 and its antitumour activity is the result of topoisomerase II inhibition.²⁸³

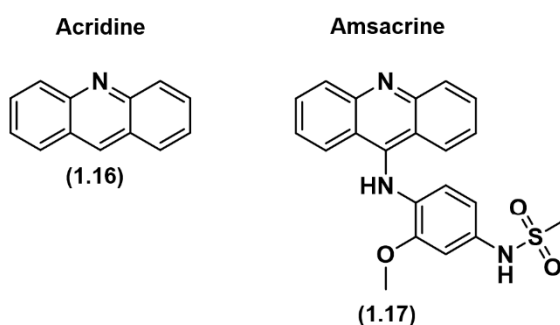


Figure 1.30: Chemical structures of acridine (1.16) and amsacrine (1.17)

Topoisomerases are a class of enzymes responsible for the release of torsional stress in supercoiled DNA.^{284–286} Cellular process like DNA replication and transcription require the local unwinding of the double helix, which must be compensated by overwinding of an adjacent region in the DNA. DNA topoisomerases introduce transient breaks in the DNA, allowing changes in the DNA topology and elimination of the overwinding. There are two types of topoisomerase (topo) enzymes, type I and type II, that introduce single-strand and double-strand breaks in the DNA,

respectively. Topo II is the target of many antitumour agents, including acridine derivatives.

As part of topo II's mechanism, the enzyme forms a covalently bound enzyme-DNA complex. Topo II inhibition by DNA intercalators, also called topo II poisons, is accomplished by stabilisation of this complex, preventing its resolution by the formation of a ternary complex. As a result, DSBs accumulate in the DNA, the stabilised topo II-DNA complex acts as a cellular toxin, blocking replication and transcription processes, leading to cell apoptosis.²⁸⁵

1.5.2 Threading intercalator 9-aminoacridine-4-carboxamide

The success of amsacrine, prompted further investigations into acridine derivatives as antitumour agents by Denny and his group, leading to the development of acridine threading intercalators.^{287–290} High DNA binding affinity, long residence times at individual binding sites and slow drug-DNA association rates were the parameters of focus for the development of more potent compounds, investigating structural alterations to fulfil these criteria.

Nogalamycin (**1.18**) is a natural product isolated from *Streptomyces nogalater*, made up of two side chains, able to bind DNA with one side chain in each groove (**Figure 1.31**).²⁹¹ The planar central scaffold of the structure intercalates into DNA, sitting between two base pairs, whilst the nogalose ring is positioned in the minor groove and the fused bicyclic sidechain lies in the major groove, stabilised by hydrogen bonds with the DNA bases.^{292,293}

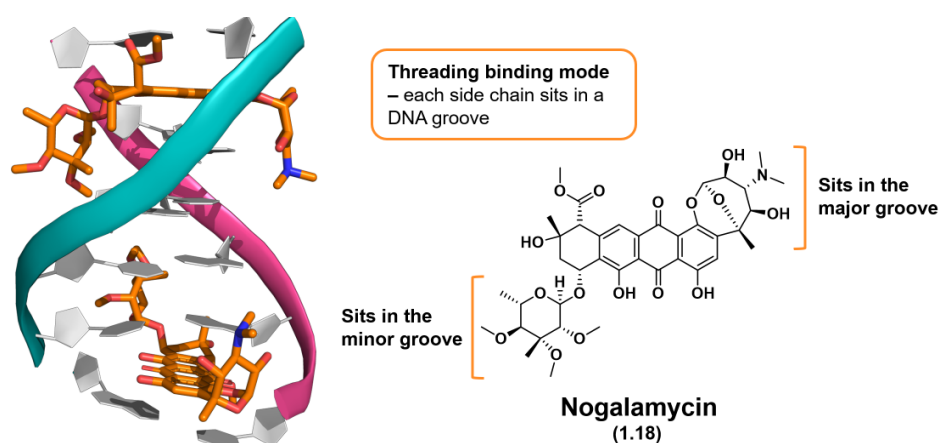


Figure 1.31: Nogalamycin chemical structure and crystal structure (PDB **182D**) of two nogalamycin molecules bound to duplex DNA through a threading mode

The described threading binding mode leads to long residence times on DNA, resulting in a highly potent RNA polymerase inhibitor.^{294–296} This led to the suggestion

that synthetic acridine analogues designed to bind similarly to nogalamycin may display potential as antitumour agents through the inhibition of transcription.

9-aminoacridine-4-carboxamides are a class of acridine derivatives designed by Denny and his group in 1984, that exhibit highly potent antitumour activity.²⁸⁷ Structural studies highlighted the significance of the carboxamide chain position, as position 2 and 3 substituted analogues displayed the same lipophilicity and similar DNA binding affinity, however, were inactive against cancer cells. Interestingly, this suggested that the antitumour activity of these compounds was not directly associated with their levels of DNA binding. Subsequent structure-activity studies revealed that any alterations in the length of the methylene section and any substitution on the NH were not tolerated, significantly decreasing the compounds activity. Wakelin later suggested that 9-aminoacridine-4-carboxamides are threading intercalators, with the acridine unit inserted between the base pairs whilst the carboxamide side chain lies in the major groove (**Figure 1.32**). A crystal structure of an acridine-4-carboxamide (**1.19**) confirmed this mode of binding, presenting the carboxamide “hooking” around the adjacent base pair, forming a hydrogen bond the neighbouring guanine base.²⁹⁷

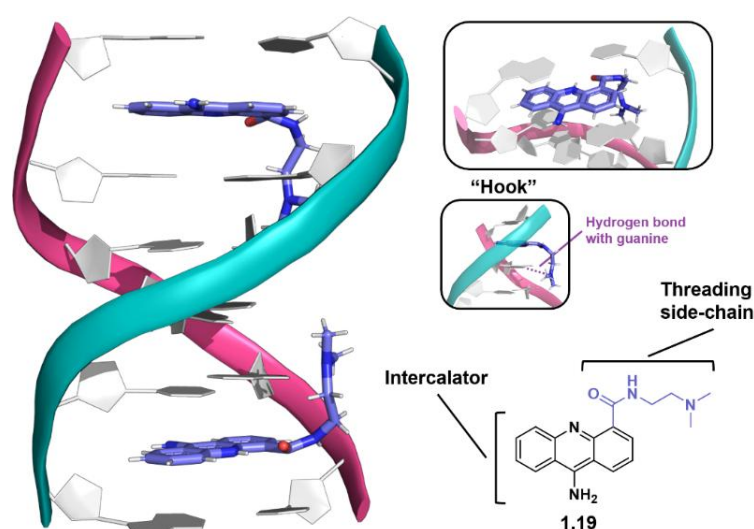


Figure 1.32: Crystal structure (PDB 452D) of two 9-aminoacridine-4-carboxamide (**1.19**) derivatives intercalated into duplex DNA, with the acridine group inserted between base pairs and the side chain hooking onto the DNA via a hydrogen bond with guanine.

1.5.3 Bisintercalation

To further improve antitumour activity and increase residence times, 9-aminoacridine-4-carboxamides were investigated for the development of bisintercalators.²⁹⁸ Bisintercalation is described as the insertion of two planar chromophores between DNA base pairs, positioning the group linking the chromophores in a DNA groove. Echinomycin (**1.20**) is a quinoxaline antibiotic, isolated from *Streptomyces echinatus*

in 1957²⁹⁹, and the first reported bi-functional DNA intercalating agent.³⁰⁰ It binds to DNA, inhibiting replication and transcription, displaying good antitumour activity. Several clinical trials were conducted with echinomycin reaching phase I and II, however none progressed into the clinic due to high toxicity and a narrow therapeutic window.^{301–308} The confirmed binding model of bisintercalation placed the quinoxaline chromophores between the DNA bases, forming a two-base-pair sandwich, with the cyclic depsipeptide situated in the minor groove (**Figure 1.33**).^{300,309,310}

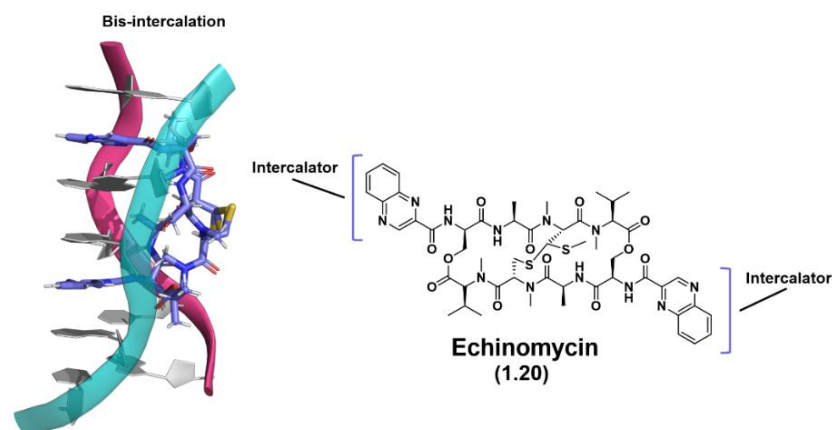


Figure 1.33: The chemical structure of echinomycin, crystal structure (PDB **2ADW**) of echinomycin bisintercalating into duplex DNA

Following the discovery of the bisintercalating binding mode of echinomycin, several studies were carried out investigating dimeric compounds bearing other chromophores. The design of naphthalimide³¹¹ and imidazolacridinone³¹² dimers presenting broad-spectrum activity in various solid tumours prompted Denny and his group to design a series of diacridines³¹³, followed by the design of the more successful bis(acridine-4-carboxamide) compounds.²⁹⁸ The designed bisintercalators exhibited higher cytotoxicity in relation to their respective monomers, confirming the hypothesis that bisintercalation yields more potent antitumour agents.^{298,314,315}

1.5.4 9-aminoacridine-4-carboxamide dimers

A new class of antitumour agents were designed based on the combination of the threading binding mode of nogalamycin and bisintercalation of echinomycin, intended to act via transcription inhibition.

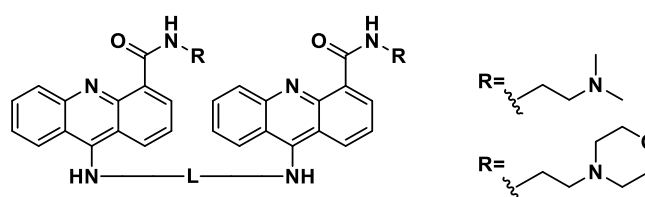


Figure 1.34: Chemical structure of 9-aminoacridine-4-carboxamide dimers designed by Wakelin et al.³¹⁶

Two 9-aminoacridine-4-carboxamide groups were linked together via the 9-amino position to yield a new series of acridine dimers.³¹⁷ The carboxamide side chain located at position 4 would thread through the DNA upon intercalation of the two aminoacridine chromophores. Two 4-carboxamide threading side chain hooks were assessed, the previously mentioned *N,N*-dimethylaminoethyl, and ethylmorpholino groups, whilst varying the nature of the linker. The dimers were intended to bisintercalate into DNA via a threading mode, with the carboxamide side chains expected to form hydrogen bonds with the O6/N7 atoms of guanine residing in the major groove, whilst the linker connecting the two aminoacridine groups is positioned in the minor groove (**Figure 1.35**). The carboxamide groups were intended to thread through the helix without hindering the aminoacridines' intercalation, making their withdrawal more difficult due to their guanine interactions, increasing the complex's lifetime. This intended binding mode was confirmed by, with the dimers' binding sites extending over four base pairs, the acridine chromophores trapping two base pairs. The most active compound identified was the C8-linked derivative, with an IC₅₀ of 50 nM against CCRF-CEM human leukaemia cells.³¹⁷

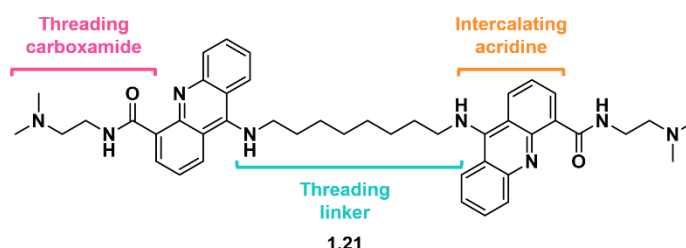


Figure 1.35: C8-linked 9-aminoacridine-4-carboxamide dimer

The acridine dimer bearing the C8 linker was reported binding to a higher-order DNA structure. A crystal structure presented the bisintercalator bound to a DNA junction-like quadruplex cavity, structurally resembling a four-way DNA junction.³¹⁸ Shortly after, a collaboration between the Searcey and Cardin groups reported the first crystal structure of a small molecule, (9-aminoacridine-4-carboxamide dimer bearing a six-carbon linker) bound to a four-way junction (**Figure 1.36**).³¹⁹ The dimer was intended to bind across the junction arms, stabilising the structure, however, a novel non-covalent mechanism was observed, with the compound bound in the middle of the junction. The acridine chromophores formed pseudo-base pairs with thymine, displacing the adenines participating in the A-T base pairs at the junction crossover.³¹⁹

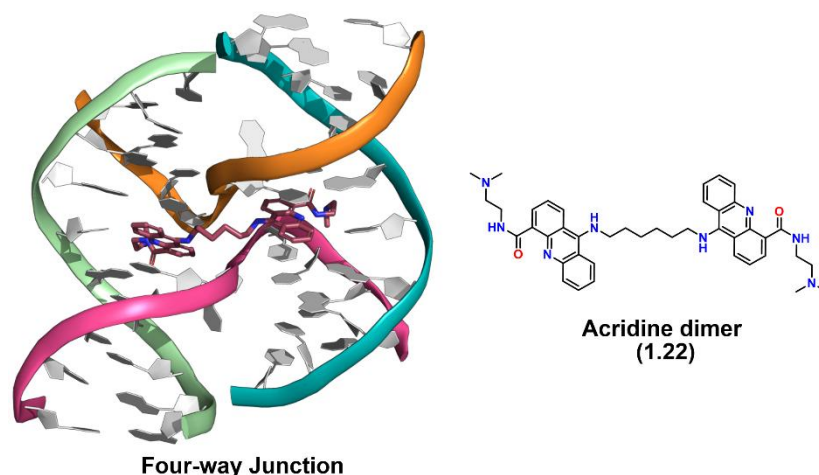


Figure 1.36: Crystal structure of a 9-aminoacridine-4-carboxamide C6-linked molecule bound at the centre of a four-way DNA junction (PDB 2GWA).

Further studies were carried out by the Searcey group, developing acridine dimers intended to bind to 4WJs through intercalation. A series of acridine dimers synthesised with “click” chemistry were reported, presenting a series of asymmetrical dimers able to bind to 4WJs.³²⁰ The position of the 4-carboxamide chain was investigated, suggesting 4WJ binding was possible when shifted to position 3. Interestingly, whilst the 9-aminoacridine-3-carboxamide dimers exhibited poor DNA binding affinity and no activity in cells, they were able to bind to 4WJs. These results highlighted the complexity and promise of 9-aminoacridine-4/3-carboxamide dimers as 4WJ binding compounds.

1.5.5 Conclusions

DNA junctions provide an interesting target for developing novel anticancer therapies, however little is known about how these structures can be controlled and selectively targeted. Three-way junctions have been extensively studied in association with trinucleotide expansion repeats which have been linked to over 40 human disorders, selective targeting of these structures could lead to the development of new therapeutic tools.^{215,219,221} Additionally, four-way junctions form during inverted repeat sequences, which have been associated with genomic instability, one of the main hallmarks of cancer.⁴⁰ Furthermore, they form during the homologous recombination repair pathway, an important process that could be targeted through a 4WJ-binding small molecule, developing a chemically-induced synthetic lethality therapy.²³¹ DNA junctions provide new opportunities for designing new therapies, however, first a better understanding of the structures and how to control them is necessary.

This research focuses on developing a series of dimeric and trimeric small molecules to selectively target four-way and three-way junctions, respectively. The ability to

target DNA junctions selectively will allow these structures to be further studied, *in vitro* and *in vivo*, learning more about their conformation and how to control them. For the design of the compounds in this research, 9-aminoacridine-4-carboxamide is used as the basic component in the small molecule design, due to its strong DNA intercalating abilities.²⁸⁷ Compound **1.22** provides a good starting point showing great promise as a DNA junction binding compound. This research aims to improve its binding properties by further exploring the potential of 9-aminoacridine-4-carboxamide multimeric compounds as DNA junction binders.

1.6 Aims and objectives

1. The synthesis and characterisation of a series of 9-aminoacridine-3/4-carboxamide dimers to target four-way junctions, evaluating the effects of incorporating rigid linkers
2. The evaluation of acridine dimers in a series of biophysical studies to assess four-way junction binding properties
3. The design, synthesis and characterisation of novel acridine trimers to target three-way junctions
4. The investigation of acridine trimers in biophysical studies to confirm the design rationale and assess their three-way junction binding and selectivity properties
5. Biological evaluation of acridine dimers and trimers synthesised in the thesis, in combination with DNA repair inhibitors, on HL-60 and A375 cancer cell lines

1.6.1 Thesis outline

This thesis investigates the design of novel small molecules targeting four-way and three-way DNA junctions as antitumour agents.

Chapter 2 describes the synthesis and characterisation of twelve 9-aminoacridine-4/3-carboxamide dimers, six of which are novel. The dimers were assessed in a total of seven assays, investigating their binding interactions with DNA junctions, stability, and selectivity over duplex DNA.

Chapter 3 outlines the design, synthesis and characterisation of two novel 9-aminoacridine-4-carboxamide trimeric complexes able to bind to three-way junctions. The trimers were carried through several *in vitro* assays assessing their binding, stability and selectivity for three-way junctions.

The compounds described in chapters 2 and 3 were evaluated for cell viability and synergy with DNA repair inhibitors in cancer cells, presented in **Chapter 4**.

Chapter 2

The design and synthesis of acridine dimers to target four-way DNA junctions

2.1 Introduction

2.1.1 Four-way junction binding compounds

A series of early studies were carried out by Kallenbach and colleagues, investigating the binding of metal complexes to a four-way junction (4WJ). In 1988, evidence was presented that a compound developed by Dervan's lab,³²¹ called $\text{MPE} \cdot \text{Fe}^{\text{II}}$, was able to bind to a 4WJ at low drug-to-DNA ratios.³²² $\text{MPE} \cdot \text{Fe}^{\text{II}}$ (**2.1**) is made up of a DNA intercalator linked to an EDTA group via a short hydrocarbon chain (**Figure 2.1**). It can bind to duplex DNA and can cleave strands in the presence of peroxide and ascorbate. A footprinting assay revealed it can intercalate into and cleave a 4WJ near the central pocket.

A set of compounds called tetrapyrrolyl porphyrins (TMPyP4) (**2.2**), were also analysed for 4WJ binding. A total of five $\text{M}_2\text{TMPyP4}$ compounds, with M representing copper, nickel, zinc, cobalt or manganese (**Figure 2.1**), were investigated, the results suggesting a high-affinity binding site for the porphyrin ring system near the 4WJ's branching point.³²³ However, the exact binding mode remains unclear.

Additionally, well-known DNA dyes were assessed through a series of assays, namely a binding competition, DNA footprinting and a DNA cleavage assay. It was shown that the dyes bind to 4WJs in a different manner to duplex DNA. Stains-All dye (**2.4**) is a dye used for DNA, RNA polysaccharides and membrane phospholipids, showing a different colour for each one.³²⁴ When tested with a 4WJ a unique colour was noted, even when compared to a dsDNA molecule of the same length. Propidium iodide (**2.3**) and Stains-All dye (**2.4**) were both found to bind to central pocket of the junction.³²⁵

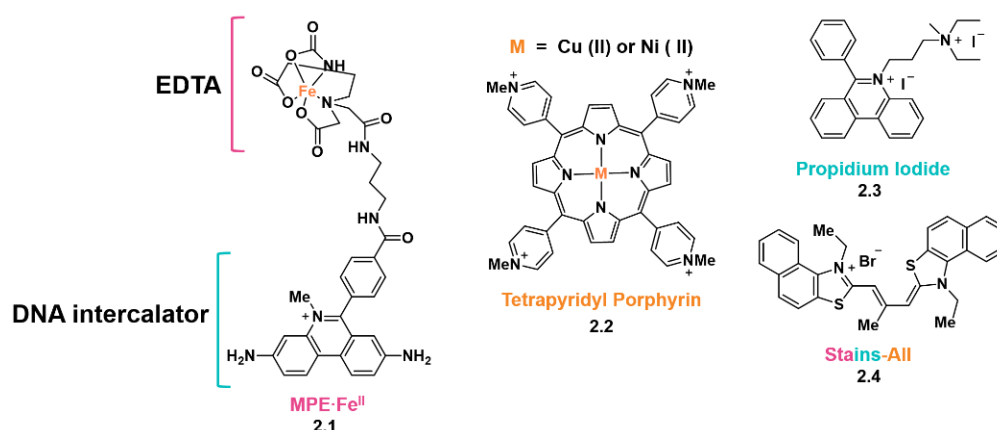


Figure 2.1: Early four-way junction targeting compounds described by Kallenbach *et al.*

2.1.2 Peptide ligands

The field of 4WJ-targeting peptides was initiated by Segall's group with the identification of promising candidates, peptides able to selectively bind 4WJs, investigating the mechanism behind their binding and biology. In trying to understand site-specific recombination pathways, a series of hexapeptides were assessed, to selectively stabilise key intermediates in the process, including the Holliday junction.³²⁶ A large-scale combinatorial screen of nearly 2.5 million hexapeptides, led to the discovery of WKHYNV (**2.5**), which can stabilise the HJ intermediate that forms in Phage λ integrase(Int)-mediated recombination (**Figure 2.2**). By binding to the HJ, the peptide blocked the resolution of the intermediate in either direction, preventing the completion of the recombination process or the regeneration of the original substrates, leading to the accumulation of 4WJ intermediates. Despite the inhibition of the recombination process, the overall growth of bacteria was not inhibited. Further investigations of the peptide showed it was also able to bind to HJ intermediates in the Cre-loxP and Flp-*frt* recombination pathways, suggesting it was unlikely to be targeting protein features but more likely the DNA structure. However, a fluorescence polarisation assay showed it was unable to bind to a protein-free 4WJ, indicating binding relies on a conformational requirement only fulfilled in the presence of a protein-bound 4WJ. Finally, a partially resolved crystal structure, placed the peptide in the poorly ordered centre of the junction.³²⁷ This was further confirmed by replacement of adenine bases with 2-aminopurine (2AP) residues in the centre of the 4WJ. 2AP residues are fluorescent, therefore the quenching that was recorded upon binding of the peptide further suggest it is binding at the centre of the junction, with the stacking of aromatic rings causing the decrease in fluorescence.

A further study was performed, focusing on the inhibition of the excision pathway in Int recombination, leading to the identification of new hexapeptides able to trap HJs, the most potent being WRWYCR (**2.6**) and WRRWCR.³²⁸ To directly compare the potency of the peptides, a value described as an IC_{50} was calculated, representing the necessary concentration of a peptide for 50% reduction in the recombination products formed. The two peptides showed a 50-fold increase in comparison to **2.5** and were also able to bind both to protein-bound HJ and protein-free HJ. Due to the cysteine residue in the peptide, it was suspected that they could form disulphide bridges and create dimeric version of themselves. To investigate whether this was the case, the experiment was repeated with dithiothreitol (DTT), preventing the formation of the bridges. The results showed an 82% reduction in the activity of the peptides, due to their dissociation from the HJ leading to their resolution. These results strongly

suggest the active form of the peptides as a dimer, assembled via a disulphide bridge between the cysteine residues of two peptide monomers. To further verify this, a dimeric version of WRWYCR was synthesised by linking the two monomers using bismaleimido-hexane (BMH), a homobifunctional cross-linking agent. The dimer showed only a slight decrease in activity and the DTT no longer had any effect, confirming the hypothesis that the peptide was only active as a dimer.

Variations of the peptide were synthesised to uncover which features were important. Shorter versions were prepared, causing a significant decrease in the peptide's affinity. An alanine replacement scan suggested only amino acids 3 and 5 were crucial to the activity of **3.6**. With this information in mind, the group investigated how the peptide was most likely interacting with the HJ intermediate through modelling of the dimer to a Cre-lox four-way junction complex (**Figure 2.2**).³²⁹ The calculated results predict both intramolecular and intermolecular stacking interactions between the WRWYCR dimer and the DNA bases located in the centre of the junction. Hydrogen bonding was also shown between the dimeric peptide with the DNA bases and the deoxyribose backbone.

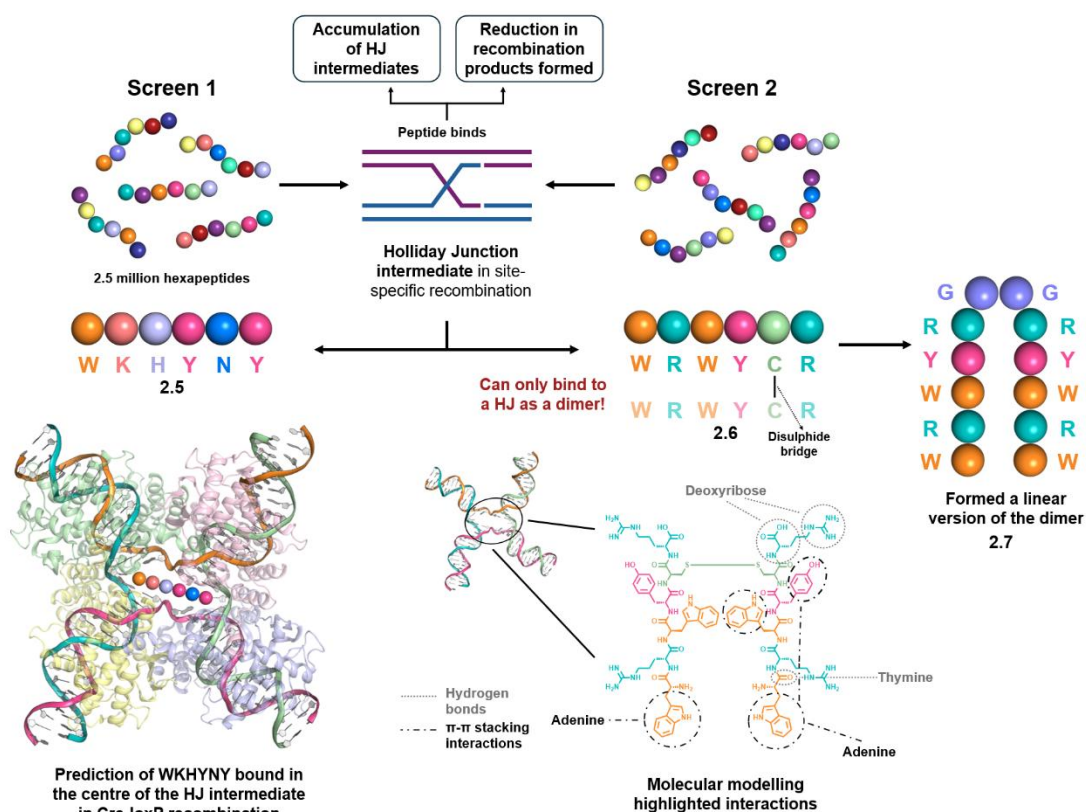


Figure 2.2: Hexapeptides identified for 4WJ binding by Segall through large-scale combinatorial screens

As the peptides were intended as recombination inhibitors, it was important to assess whether they could prevent proteins from binding to the HJ intermediate. The protein

complexes chosen where the RecG helicase and RuvABC of *E.coli*, both known to bind to the HJ. The WRWYCR dimer was able to inhibit the binding of both, and a gel mobility shift assay proved the peptide could competitively inhibit RecG binding. The inhibition is believed to be a result of both the peptide and the helicase binding to the same substrates of the HJ, therefore not allowing simultaneous binding.

A gel mobility assay was carried out in the presence and the absence of Mg^{2+} , which respectively results in the X-stacked or open conformation of the 4WJ.³³⁰ The peptide was unable to bind in the presence of Mg^{2+} , confirming preference for the open form, believed to be due to the square-planar conformation providing more space in the central pocket for peptide binding. A competition assay was carried out measuring the peptide's selectivity for 4WJ binding over non-specific duplex DNA. An excess of dsDNA was added, showing high selectivity for the 4WJ in the presence of up to 240-fold dsDNA.

Peptide **2.6** was assessed for antimicrobial activity, the results indicating activity in both Gm- and Gm+ bacteria, with a slight increase in the latter.³³¹ The D-isoform of the peptide, wrwycr, was also evaluated, the results showing higher bacterial inhibition than the L-isoform, possibly due to higher resistance against cellular peptidase degradation, compared to the L-isoform. The wrwycr peptide trapped the 4WJs, preventing the repair of collapsed replication forks and therefore leading to cell death.³³² Both isoforms were also able to enter the bacterial cell to inhibit site-specific recombination *in vivo*. An experiment was carried out measuring bacterial growth inhibition in the presence of mammalian cells. The results gathered showed that the peptides had a significant effect on the growth of the bacterial cells, whilst displaying no toxicity towards the mammalian cells. The displayed selectivity holds promise in developing novel antibiotics directly targeting DNA repair pathways.

Moving forward, the group synthesised a single-chain linear version of the WRWYCR peptide, maintaining its characteristics whilst eliminating the need for dimerization, as the disulphide bridge, necessary for the activity of the peptide can be reduced in the cell, making it unreliable. WRWYRGGRYWRW (**2.7**) was synthesised as a linear analogue.³³³ Both D and L-isoforms of this peptide were able to bind to protein-free 4WJs, trap HJs in site-specific recombination and inhibit the activity of DNA repair enzymes acting on the junction. The D-isoform showed better 4WJ binding. Even though wrwyrggrywrw measured higher non-specific DNA binding when compared to wrwycr, when looking into its antimicrobial activity, it showed similar or increased inhibition in Gm- and Gm+ bacterial cell growth.³³³

2.1.3 Macrocycles

Following the success of the peptides described in **section 2.1.2**, a screen of macrocycles based on their characteristics was carried out.³³⁴ In the initial screen, eight macrocycles were assessed (**2.8**), their design inspired by the cocrystal structure of the WRWYCR dimer, considering the symmetrical nature of a HJ. The final hexameric cyclic compounds (**2.8**) were more rigid than their lead linear peptides and approximately the size of the HJ binding pocket. An assay measuring RuvC endonuclease inhibition in *E. coli* was used to assess their 4WJ binding abilities. The results gathered suggest three of the compounds could trap 4WJs when in the open conformation.

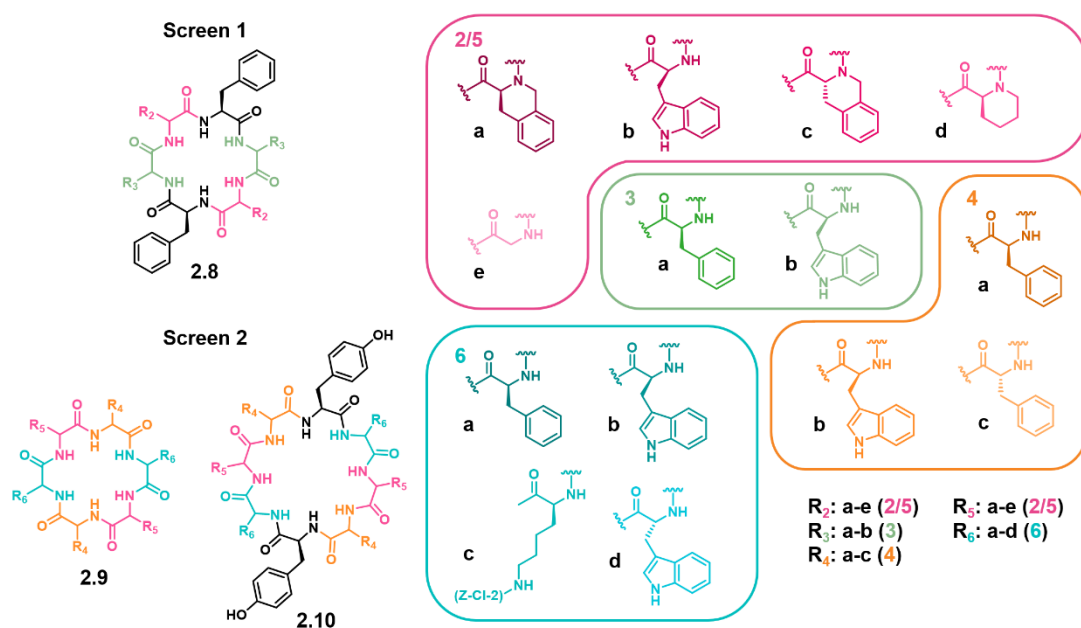


Figure 2.3: Cyclic peptides identified by Segall et al. from screen 1 and 2 for 4WJ binding

The second screen aimed to simplify the synthetic approach used for the peptide synthesis, as well as design macrocycles that provide flexibility in amino acid placement.³³⁵ Regarding the residue composition, hydrophobic residues previously shown to be significant when trapping HJs were maintained whilst also incorporating hydrophilic residues. From a total of eighteen compounds (**2.9**, **2.10**), eleven were tested in the RuvC endonuclease-based assay, indicating that any peptide including a tyrosine at any position could trap 4WJs. Both hexameric (**2.9**) and octameric (**2.10**) peptides were assessed, showing that octamers were accommodated in the central pocket of a 4WJ, in some cases showing better 4WJ trapping than their respective hexamers. The cyclic peptides were tested against gram negative and positive bacteria, however observing no effect on the growth of either.³³⁶

2.1.4 Acridine-based compounds

Acridine dimers were first studied in relation to 4WJ binding by Carpenter *et al.*, designing dimeric structures to probe DNA structures.³³⁷ The final compounds were made up of two acridine (**2.11** – **2.14**) or phenanthridine (**2.15**, **2.16**) moieties, connected by a rigid linker, pointing them outwards towards opposite directions (**Figure 2.4**). The compounds' binding properties were assessed via a DNA cleavage assay. DNase I was used to monitor whether, and where, the compounds were binding to the designed 4WJs. The results suggest that APEPA (**2.11**) could bind to 4WJs, however, details on the mechanism of action or the site of binding were inconclusive.

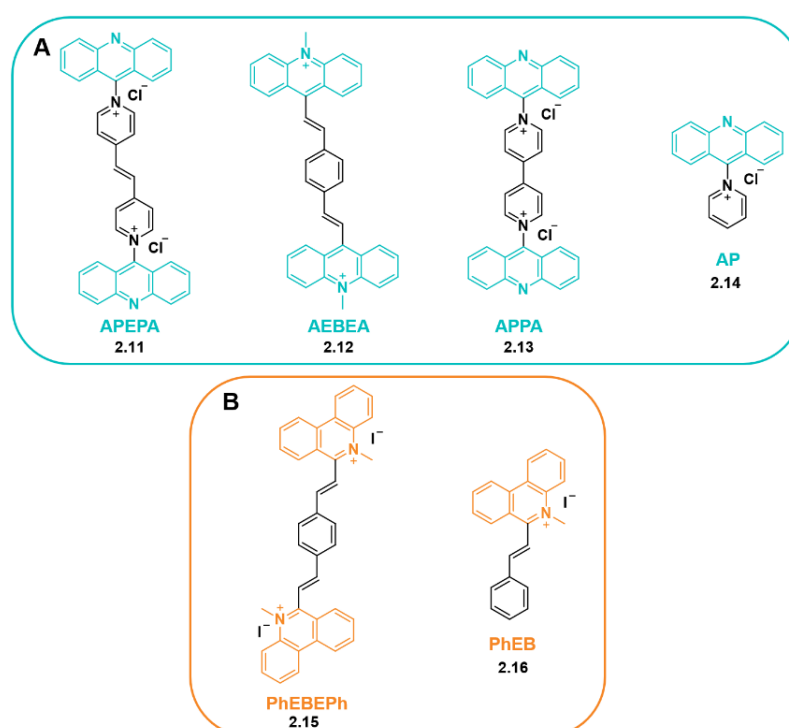


Figure 2.4: Dimers studied by Carpenter *et al.* bearing two **A.** acridine or **B.** phenanthridine moieties

Dimeric structures made up of 9-aminoacridine-4-carboxamide have been extensively studied by the Searcey group as 4WJ binding compounds. The strategy is based on the ability of the dimers to bind across two duplex arms of the junction simultaneously via the two intercalating groups. An X-ray crystallographic study was carried out by the group in collaboration with Prof Cardin, investigating the interaction of a 9-aminoacridine-4-carboxamide dimer (**2.18**) and duplex DNA.³³⁸ The results showed one acridine-4-carboxamide group intercalating into one duplex, whilst the other was independently bound to another duplex in a symmetry-related manner, (**Figure 2.5**). This duplex-to-duplex DNA crosslinking inspired the 4WJ binding strategy.

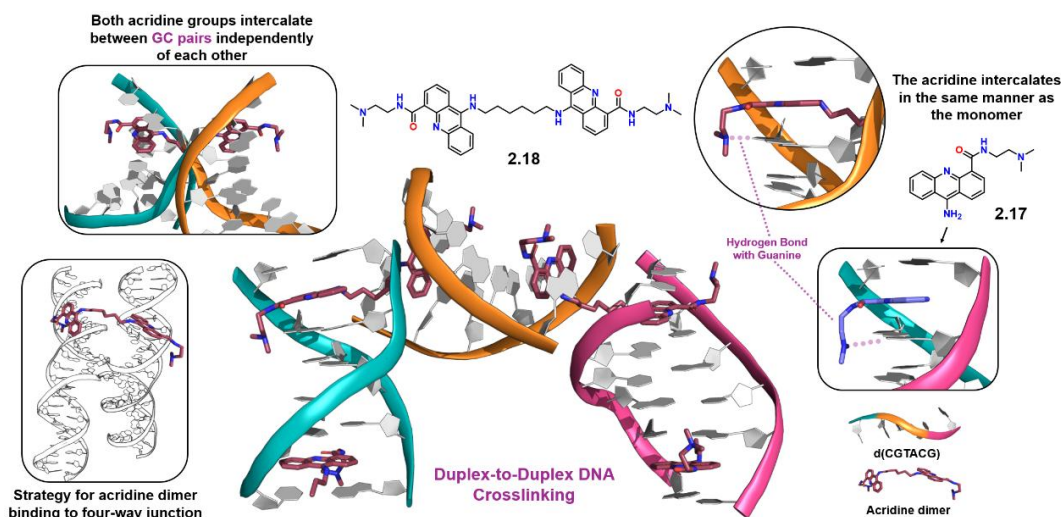


Figure 2.5: X-ray crystallography of an acridine dimer with d(CGTACG), carrying out duplex-to-duplex DNA crosslinking, described by Searcey and Cardin (PDB 2GB9)

Shortly after, the group acquired the first crystal structure of a small molecule bound to a 4WJ, presented in **Figure 2.6**.³¹⁹ The structure demonstrated acridine dimer **2.18**, non-covalently bound across the centre of an X-stacked 4WJ. To allow for the bis-acridine to sit in the central pocket of the 4WJ, two adenine bases were flipped out and replaced by the acridine moieties, forming pseudo-base pairs with the two thymine bases. The binding is stabilised by π - π stacking interactions and hydrogen bonding between the amide-hydrogen found on the acridine's side-chain and the oxygen on the cytosine base located near the central pocket.

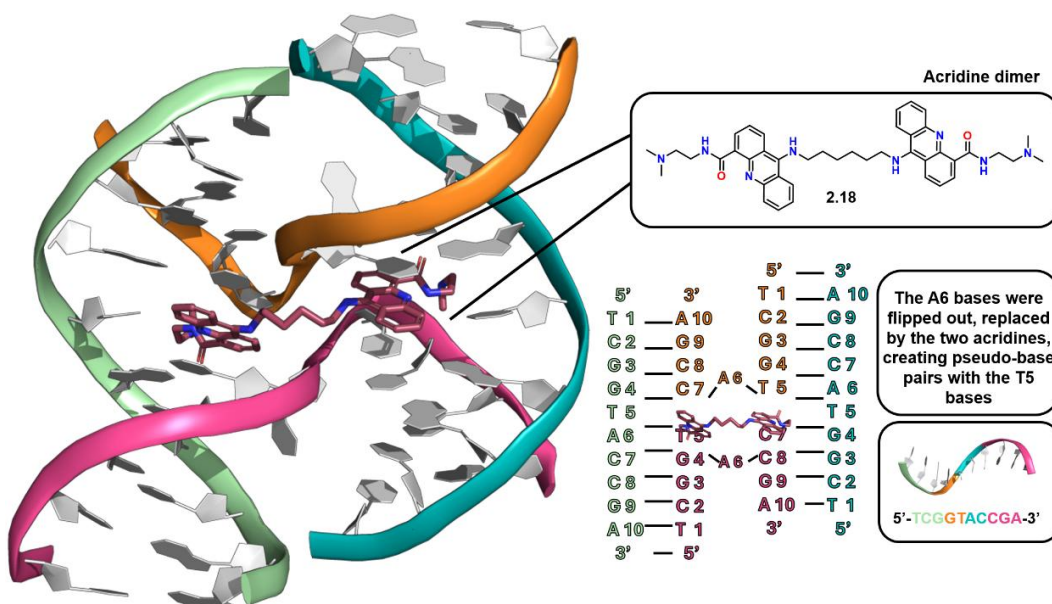


Figure 2.6: Crystal structure of C6 acridine dimer bound to the centre of a 4WJ, displacing two adenines and forming pseudo-base pairs with two thymine bases (PDB 2GWA)

The discovery of this new mode of binding led to the investigation of more acridine dimers as 4WJ-binding compounds. A small library of acridine dimers (**Figure 2.7**)

was synthesised using “click” chemistry.³²⁰ Variations of acridine monomers bearing an alkyne or azide were synthesised, whilst also alternating the side-chain’s location between positions 3 and 4. All combinations were assessed by gel electrophoresis experiments, the results suggested only **2.20** and **2.21** were able to bind to 4WJs.

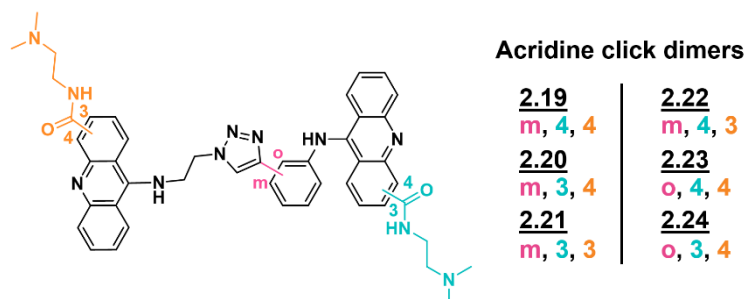


Figure 2.7: A small library of acridine dimers synthesised with “click” chemistry, o/m described whether the triazole is ortho or meta couples to the amine, 3/4 described the position of the carboxamide chains for each acridine

A set of acridine monomers synthesised with “click” chemistry were further investigated, leading to the discovery of RTA (**2.25**), a 9-aminoacridine-3-carboxamide monomer with a short, chain at the 9-position, presented in **Figure 2.8**.³³⁹ Prior to this compound, to fold an open conformation 4WJ to its X-stacked form, high temperatures were necessary in the presence of Mg^{2+} . Compound **2.25** is the first example of a compound able to promote the formation of an X-stacked 4WJ at room temperature. These results were confirmed via CD and gel electrophoresis experiments. The ability to control the conformation of a 4WJ at room temperature could prove an important tool in regulating and folding 4WJ structures in cells.

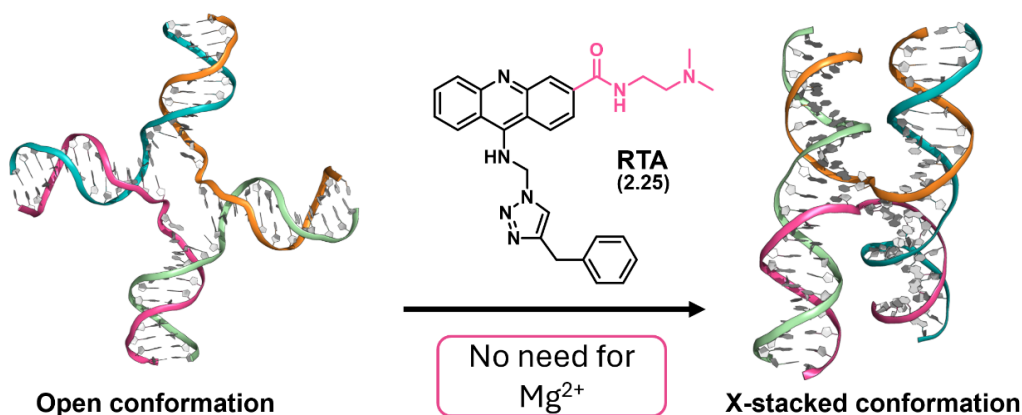


Figure 2.8: Acridine monomer RTA can induce the X-stacked conformation of a 4WJ in the absence of Mg^{2+}

With RTA as a starting point, a series of acridine monomers were synthesised to target 4WJs (**Figure 2.9**), altering the phenyl unit of **2.25** with a selection of aryl groups. Three new compounds were identified, **2.39**, **2.40** and **2.46**, maintaining the carboxamide side-chain at position 3, bearing a slightly shorter linker between the

acridine and the triazole. All three compounds bind to 4WJs at room temperature, whilst only **2.39** and **2.40** display antiproliferative activity, making it hard to pinpoint their mechanism of action in cells.

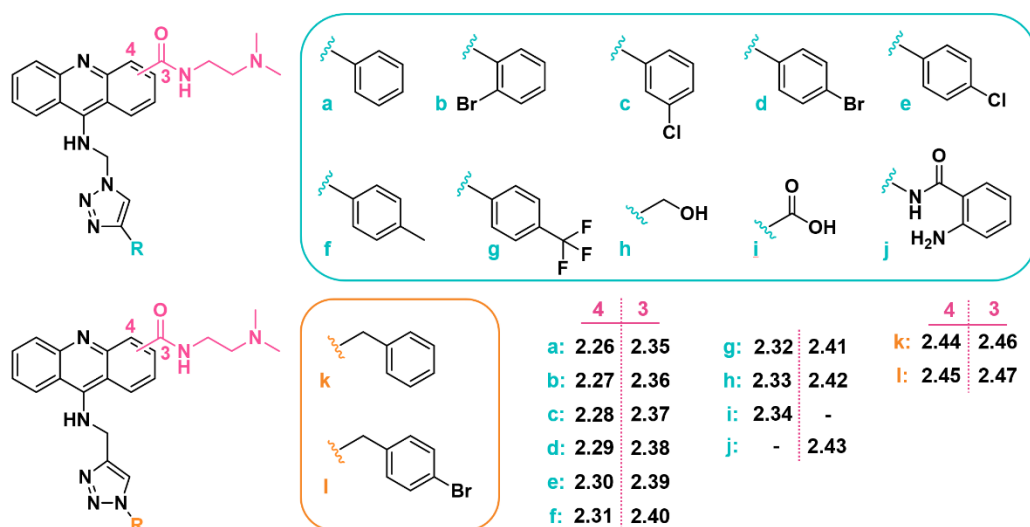


Figure 2.9: A library of acridine monomers synthesis using “click” chemistry

2.1.5 Triaminotriazine-acridine conjugate

More recently, Chien *et al.* disclosed a crystal structure of a triaminotriazine-acridine conjugate (**2.48**) bound to a non-canonical 4WJ, described in **Figure 2.10**.³⁴⁰ Compound **2.48** is a known DNA binder and exhibits strong binding affinity to CTG trinucleotide repeat sites, containing T:T mismatches, previously investigated for the treatment of myotonic dystrophy type 1. The binding mode proposed relies on the triaminotriazine group recognising a T:T mismatch and either forming a base triplet or flipping out one of the pyrimidine bases to form a Watson-Crick-Franklin like pseudo-base pair, whilst stacking on the acridine.

The group crystallised **2.48** with a palindromic duplex containing three consecutive CTG repeats. The resolved structure showed two **2.48** compounds intercepting the DNA duplex at both ends, leading to major structural changes and the assembly of two DNA duplexes into a non-canonical 4WJ. The two duplex molecules perform a U-turn and adopt a head-to-head topology, allowing a crossover of each chain at the junction site. The **2.48** compounds are sat in the middle of two overlapping -GCTGC-sequences, with the triaminotriazine stacked over the acridine, and the acridine group stacking with a guanine base. For the triaminotriazine unit to fit between the base pairs and allow the DNA to bend, the thymine bases in that position were flipped out.

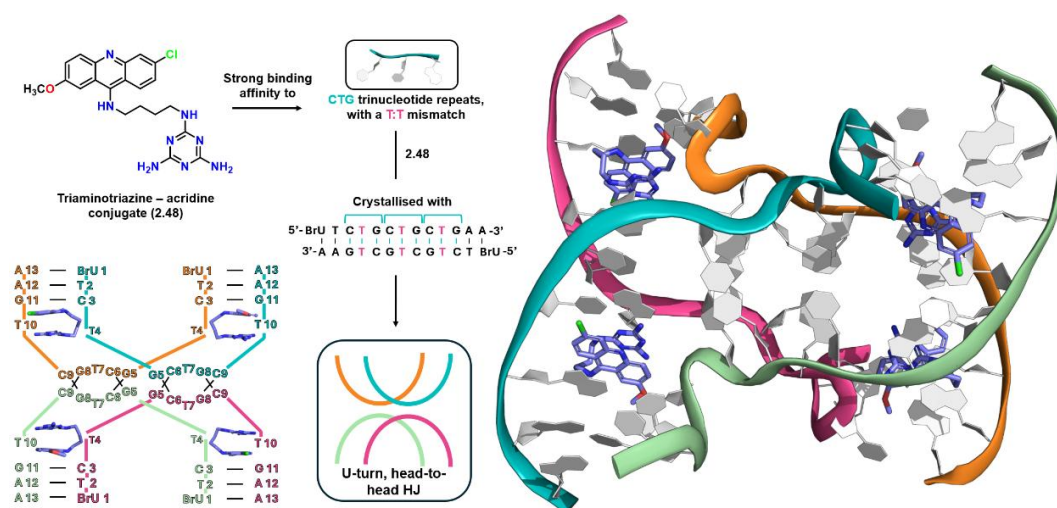


Figure 2.10: Crystal structure of triaminotriazine-acridine conjugate **2.48**, inducing the formation of a non-canonical 4WJ complex (PDB 6M4T)

2.1.6 Cyclic thiourea and guanidines compounds

Due to limitations regarding the drugability of peptides discussed in **section 2.1.2**, such as their poor permeability, Segall and co-workers carried out a screen to identify small molecules that hold similar HJ-binding abilities.³⁴¹ A total of 11 small molecule libraries, containing over 9 million compounds were screened. An *N*-methyl aminocyclic thiourea (**2.49**) was identified, **Figure 2.11**, able to bind to the HJ intermediate and inhibit *in vitro* recombination assays, causing high accumulation of HJs as well a reduction in recombination products. The compound was also able to bind to protein-free 4WJs, as well as inhibit HJ-processing helicases from binding. However, when tested in bacterial cells, no activity was observed.

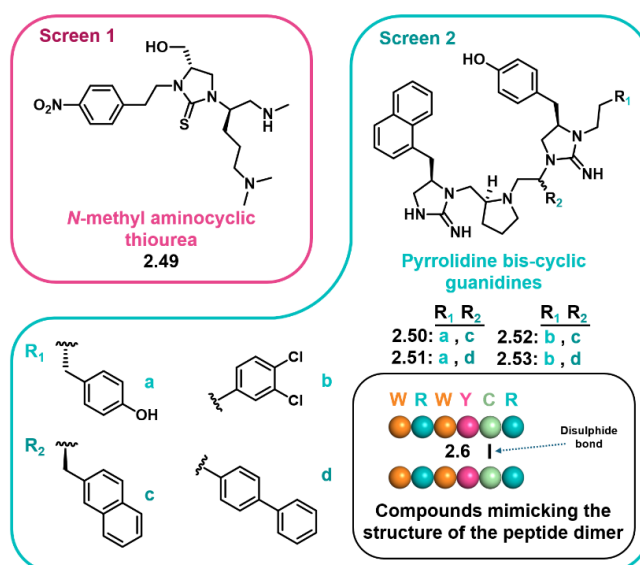


Figure 2.11: Small molecule screen carried out by Segall, identifying *N*-methyl aminocyclic thiourea and a series of pyrrolidine bis-cyclic guanidines

Aiming to identify more suitable small molecules, another screen focussing on larger compounds of a symmetrical nature was performed, mimicking the WRWYCR dimer peptide(**2.6**).³⁴² A group of pyrrolidine bis-cyclic guanidine compounds (**Figure 2.11**) showed promise, inhibiting site-specific recombination. Compound **2.52** was further tested against protein-free 4WJs, displaying moderate binding, lower than **2.6**. The compound has good antibacterial activity against both gram positive and negative bacteria, with higher potency than the peptides described in **section 2.1.2**. Some concerns were noted regarding the detected toxicity of compound **2.52** in eukaryotic cells.

2.1.7 Platinum complexes

Inspired by the success of cisplatin, a platinum complex that targets duplex DNA used in the clinic, various studies have been carried out investigating the ability of Pt complexes to bind to different DNA structures. Bonnet *et al.*, reported the induction of a 4WJ-like structure by a mononuclear platinum complex, [Pt(H₂bapbpy)]PF₆ (**2.55**).³⁴³ This compound was crystallised with the self-complementary hexanucleotide sequence 5'-CGTACG-3' (**Figure 2.12**), previously used in three-way junction studies.

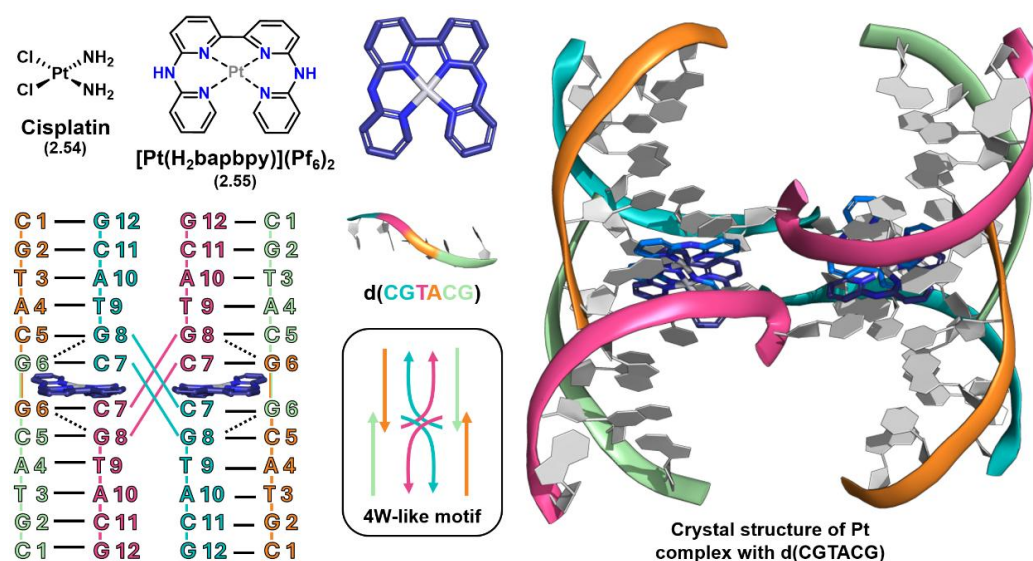


Figure 2.12: Crystal structure of [Pt(H₂bapbpy)](PF₆)₂ forming a 4WJ-like complex (PDB 6F3C)

The resolved structure showed that the presence of **2.55** led to the assembly of four oligonucleotides into a structure resembling a four-way junction. Interestingly, the distortion caused to the helices created a binding pocket for the compound made up of two tetrads. Its antiproliferative activity was assessed in cancer cell lines, including a cisplatin-resistant cell line, to help uncover its mechanism of action. Compound **2.55** was able to enter the nucleus and interact with nuclear DNA, the results showing higher potency than cisplatin. A large part of the activity was retained in the cisplatin-

resistant cells, indicating a different mode of action. However, **2.55** was also able to interact with duplex DNA, therefore this could also play a role in its biological activity.

2.1.8 Compound VE-822

Recently, Wang *et al.*, carried out an in-lab library screen to identify new 4WJ stabilisers. A total of 160 DNA damage and repair related compounds were screened in an EMSA, the results highlighting VE-822 (**2.56**) as a promising candidate.³⁴⁴ VE-822 (**Figure 2.13**) is a known ATR inhibitor, ATR proteins are key regulators in the DNA damage response (DDR) pathway, investigated as an anti-cancer agent. The compound was able to stabilise HJs upon annealing as shown through an EMSA assay and a fluoresce quenching assay. The latter assay works on the basis of two fluorescent tags being brought together upon formation of a HJ and FRET occurring between them, which can be monitored. These two assays were repeated at 37 °C, the results showing that the compound can also promote the formation of HJs in the absence of annealing too. Additionally, to directly investigate the relevance of this ability *in vivo*, the double-labelled HJ was transfected into cancer cells, where the results showed that VE-822 can promote the assembly of HJs in cells. Looking deeper into the mode of binding, a combination of CD experiments and computational modelling indicate the compound is shifting the HJ to the X-stacked conformation. Further *in vivo* assay results show good antiproliferative activity in siATR cells and that VE-822 reduces homologous recombination levels, leading to an increase in DNA damage and, ultimately, cell death.

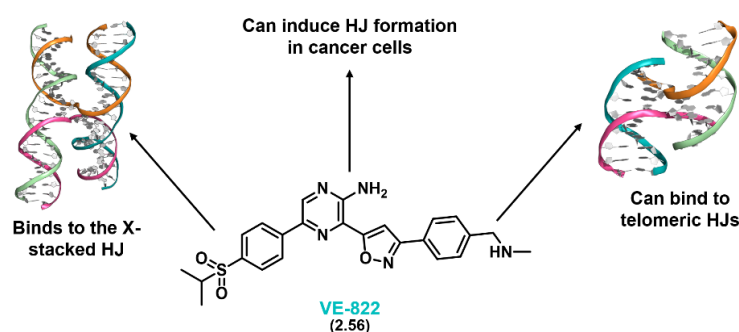


Figure 2.13: ATR inhibitor VE-822 binds to a four-way junction in the X-stacked conformation

The group carried out research investigating whether the compound can be used to target telomeric 4WJs. A 4WJ intermediate forms in the alternative lengthening of telomeres (ALT) pathway, a pathway manipulated by cancer cells to avoid telomere shortening, allowing cells to keep dividing and leading to cancer cell immortality. Therefore, targeting the 4WJ intermediate and ultimately blocking this pathway could be a promising therapeutic approach. Results exhibited that VE-822 can bind to telomeric 4WJs, however with 3-fold less potency to free 4WJs. Further assays

demonstrated that the compound could assemble telomeric-4WJs, causing telomeric DNA damage. A synergy assay was carried out with doxorubicin in osteosarcoma cells, which are known to rely on the ALT pathway, indicating that the cells were sensitised to doxorubicin upon treatment with VE-822.

2.1.9 4'-Hydroxymethyl-4,5',8-trimethylpsoralen (HMT)

Eichman *et al.* presented a crystal structure of 4'-hydroxymethyl-4,5',8-trimethylpsoralen (**2.58**) (HMT), bound to a 4WJ, shown in **Figure 2.14A**.³⁴⁵ HMT is a type of psoralen (**2.57**), a type of compound used in photochemotherapeutic treatments for skin diseases, able to cross-link and covalently modify duplex DNA.³⁴⁶ The group were interested in studying the cross-linking effect on the conformation of DNA by crystallising an HMT-DNA adduct, using the DNA sequence d(CCGCTAGCGG). Interestingly, the resolved crystal structure showed the formation of a 4WJ, with two bound HMT compounds. Two HMT-crosslinked duplex DNA molecules had assembled in the crystal in a symmetrical manner, forming a non-canonical antiparallel x-stacked 4WJ structure. The compound was further crystallised with a previously studied 4WJ-forming sequence, d(CCGGTACCGG), depicted in **Figure 2.14B**. HMT could successfully bind to the 4WJ, having little effect on the structure's conformation, when compared to the unbound form⁴⁰.

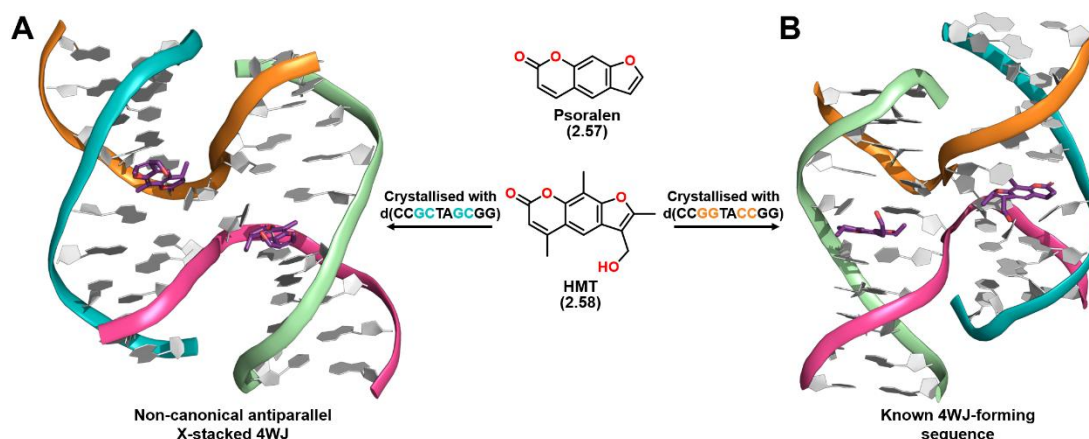


Figure 2.14: **A.** Crystal structure of HMT bound to **A.** a non-canonical 4WJ formed by d(CCGCTAGCGG) (PDB 1FHY), **B.** a known-4WJ forming sequence d(CCGGTACCGG) (PDB 1FHZ)

2.1.10 Organometallic complexes

Prof Hannon's group have carried out extensive research in 3WJ-binding compounds, having identified and optimised several metallohelicates. The group have studied the structure of a 3WJ, identifying possible binding sites, as well as pinpointing key features junction-binding compounds should bear, all of which will be discussed in **Chapter 3**. With all these findings in hand, they adjusted certain factors to switch their target to 4WJs.³⁴⁷ These requirements were met by a supramolecular organometallic

complex called a pillarplex (**2.59**). It is made up of two macrocyclic ligands comprised of six aryl rings, linearly coordinated by either eight gold or silver centres (**Figure 2.15**). A polyacrylamide gel electrophoresis (PAGE) assay was carried out to assess 4WJ binding, the results noted a shift of the 4WJ band slightly higher than the control band, suggesting **2.59** was binding to the junction, causing the complex to move slower on the gel. To gain some more information on the potential binding mode, molecular docking was completed, as both crystallisation and NMR analysis were unsuccessful. The resulting structures placed **2.59** in the middle of the central pocket of an open-form four-way junction, with the adjacent base-pairs repositioning to fit the compound. A gel competition assay comparing the pillarplex's preference for duplex DNA, 3WJ or 4WJ showed a clear preference for Y-forks and 4WJ, exhibiting a higher preference for the 4WJ between the two. Cytotoxicity studies in A549 cells showed moderate cytotoxicity for the Ag pillarplex at 24 hours post exposure, and the Au pillarplex at 72 hours.

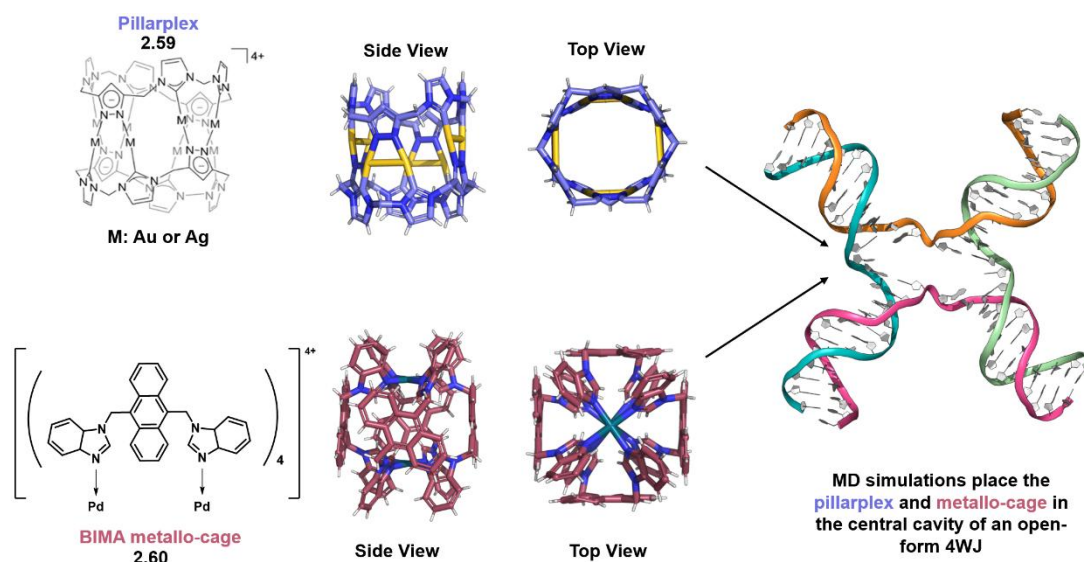


Figure 2.15: A pillarplex complex and a BIMA metallo-cage described by Hannon as 4WJ-binding compounds

Molecular docking results suggested the size of **2.59** was too small for the central cavity of the junction, potentially making the binding unstable. Therefore, the group turned to available M_2L_4 complexes, a complex motif similar to that followed for the 3WJs (M_2L_3), to identify a better fit. The requirements were met by a palladium (II)/platinum cage (**2.60**) formed by a bis-benzimidazole ligand bearing an anthracene spacer (BIMA).³⁴⁸ The metallo-cage holds a square tubular shape, with similar proportions to the junction pocket (**Figure 2.15**). Additionally, the tetracationic nature of the complex may aid binding to DNA, whilst the anthracenes located on the linkers are the ideal size for stacking interaction with the base-pairs found in the junction.

To assess whether the complex held any potential as a 4WJ binding compound, molecular docking was carried out. The results showed the metallo-cage fits well in the junction pocket of a 4WJ, when in the open-form conformation. Taking this into *in vitro* experiments, a PAGE assay was performed, where upon addition of the complexes a clear 4WJ band appears on the gel, as they are stabilising the structure. The assay was repeated in the presence of Mg^{2+} , resulting in a large amount of the 4WJ adopting the X-stacked conformation, weaker or no binding is observed. A competitive PAGE assay was also presented, a mixture of 4WJ and Pt-BIMA was titrated with increasing amounts of different DNA structures to assess the compound's selectivity. By quantification of the intensity of the 4WJ band a clear preference for 4WJs was noted, followed by a Y-fork and then a 3WJ. No competition was measured in the presence of duplex DNA. The stability of the 4WJ-Pt-BIMA complex was assessed via UV melting, and binding constants were calculated by microscale thermophoresis.

2.1.11 Conclusions

Motivation for the study described in this chapter, considering the four-way junction binding compounds discussed in this section, was inspired by the promise of the peptides and acridine-based small molecules in targeting 4WJs. The peptides described by Segall show great promise as 4WJ binders, with a high selectivity for 4WJs over duplex DNA (240-fold).^{326,328,333} However, one of the main drawbacks of peptides being used as therapeutics is their cell membrane permeability.^{349,350} In comparison, small molecules rarely encounter permeability issues and therefore are often preferred for the development of new therapeutics.

Acridine dimer **2.18** was the first small molecule crystallised bound to a 4WJ, showing great promise as a DNA junction binder.³¹⁹ Interestingly, the presented binding mode was junction binding with the displacement of two DNA bases to allow the compound to fit, not intercalation. Compound **2.18** bears two 9-aminoacridine-4-carboxamide groups, which are strong DNA intercalators, able to bind to more than one DNA molecule at the same time.³³⁸ The work described in this chapter aims to combine these modes of binding, altering the structure of **2.18** to encourage 4WJ binding through duplex-to-duplex DNA crosslinking across the DNA junction, rather than junction binding. The absence of defined structural components of the centre of the junction makes selective targeting a challenge, therefore the intended binding mode for the synthesised compounds intercalation across the 4WJ.

2.2 Chapter aims

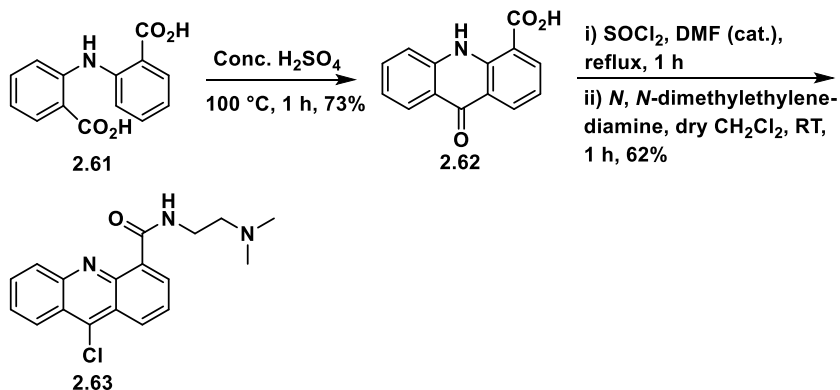
At the outset of this project, acridine dimers represented a compound class that had high affinity for 4WJ DNA, albeit with minimal selectivity for 4WJs over dsDNA. Having demonstrated 9-aminoacridine-3/4-carboxamide could crosslink DNA to DNA, duplex to duplex, promote the formation of 4WJ DNA and potentially bind to form a novel structure, the aim was to design molecules to increase 4WJ selectivity over dsDNA.

When designing the acridine dimers in the thesis, a key element was their ability to form duplex-to-duplex crosslinks, as this suggests a potential to bind to X-stranded 4WJ forms, locking the 4WJ in place. This design could also contribute to a more rigid structure binding in the mode at the junction cross-over, displacing an adenine. With this in mind, a total of twelve dimers were designed and synthesised. The dimers were subsequently evaluated in a series of biophysical assays, investigating their DNA binding properties, determining their 4WJ-binding and stabilising ability and evaluating their selectivity for binding to DNA junctions over duplex DNA.

2.3 Results and discussion

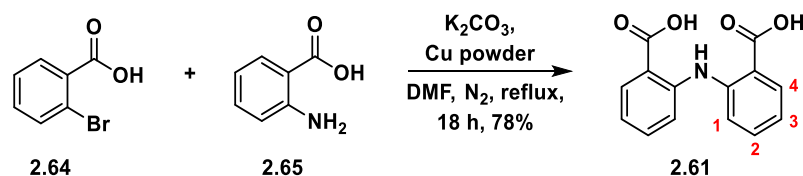
2.3.1 Synthesis of 9-chloroacridine-4-carboxamide monomer (2.63)

The 9-aminoacridine-4-carboxamide monomer **2.63** was synthesised following a published procedure, summarised in **Scheme 2.1**.^{351,352} Commercially available 2,2'-iminodibenzoic acid (**2.61**) was converted into 9-oxo-9,10-dihydroacridine-4-carboxylic acid (**2.62**), more commonly referred to as acridone-4-carboxylic acid via a Jourdan-Ulmann copper-catalysed reaction. This was followed by conversion into 9-chloroacridine-4-carbonyl chloride, before amination with *N,N*-dimethyl-ethylene diamine to generate the desired sidechain on the 9-chloroacridine-4-carboxamide monomer **2.63**.



Scheme 2.1: Synthetic pathway of 9-aminoacridine-4-carboxamide (**2.63**)

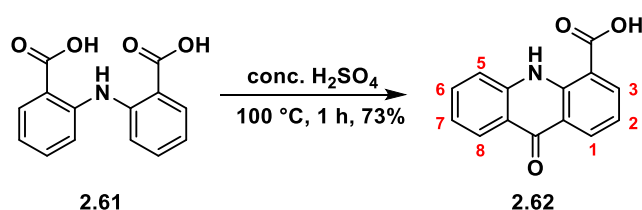
2,2'-iminodibenzoic acid (**2.61**) is commercially available therefore its synthesis was only briefly explored. Following original methods described by Rewcastle *et al.*, the dibenzoic acid was prepared via a Jourman-Ullman copper-catalysed reaction, between an aromatic amine and a 2-bromobenzoic acid.³⁵²



Scheme 2.2: Jourman-Ullman copper-catalysed reaction between 2-bromobenzoic acid (**2.64**) and anthranilic acid (**2.65**), with potassium carbonate and copper powder

Reactants 2-bromobenzoic acid (**2.64**) and anthranilic acid (**2.65**) were heated under reflux in dimethylformamide (DMF), in the presence of potassium carbonate and copper overnight. Upon completion of the reaction, it was quenched with cold water and activated charcoal, which was removed along with the copper powder via a celite filtration. The filtrate was acidified with hydrochloric acid leading to precipitation, which was collected and dried, giving a yield of 78%.

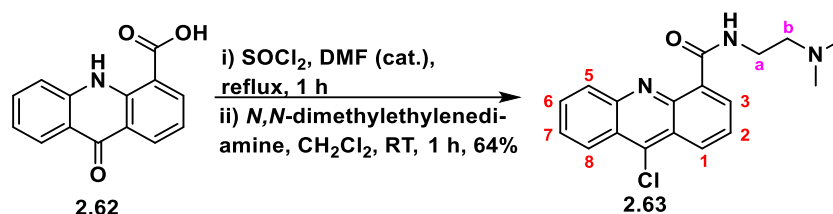
When analysing the ^1H NMR spectrum of **2.61**, there are a total of three signals, all located in the aromatic region, integrating to a total of eight protons. A doublet of doublets located at 7.9 ppm, the most downfield signal, corresponds to proton 4 (**Scheme 2.2**), due to its proximity to the electron-withdrawing carboxylic acid. Its multiplicity is a result of coupling to proton 3 with coupling constant (J) of 7.9 Hz and a smaller coupling to proton 2 with $J = 1.5$ Hz. A doublet of doublet of doublets at 6.9 ppm integrating to two, was matched to proton 2, coupled to protons 3, 1 and 4 with coupling constants 7.9, 6.4 and 1.5 Hz accordingly. Finally, a multiplet at 7.4 ppm, integrating to four protons, is made up of two overlapping signals corresponding to protons 1 and 3. A total of seven signals are observed in the ^{13}C NMR spectrum due to the symmetry of the compound. The signal found most downfield at 168.8 ppm corresponds to the carbonyl carbon, its binding to two oxygen atoms causing its shift. The high-resolution mass spectrum recorded a signal at 258.0776, matching the calculated $[M+H]^+$ ion, 258.0766.



Scheme 2.3: Cyclisation of acridone-4-carboxylic acid (**2.62**) with concentrated sulphuric acid

Compound **2.61** was cyclised following a procedure published by Denny, by refluxing in concentrated sulphuric acid for one hour, followed by an acid-base workup.³⁵¹ The product was obtained at a 73% yield as a bright yellow powder.

The ^1H NMR spectrum of **2.62** confirmed the formation of the desired product, decreasing the total number of protons from eight to seven (**Scheme 2.3**). Two doublets of doublets corresponding to protons 1 and 8 are found at 8.52 ppm and 8.44 ppm accordingly, located the furthest downfield due to their proximity to the carbonyl group at position 9. The signals appear as doublets of doublets as proton 1 is *ortho*-coupled to proton 2 ($J = 8.0$ Hz) as well as long range *meta*-coupled to proton 3 ($J = 1.6$ Hz). The same pattern is observed for proton 8 regarding protons 7 ($J = 7.4$ Hz) and 6 ($J = 1.8$ Hz), respectively. The signals for proton 3 appears as a broad doublet at 8.23 ppm due to its proximity to the electron-withdrawing, deshielding amide carbonyl and *ortho*-coupling to proton 2. Two multiplets are observed at 7.75-7.79 ppm and 7.30-7.38 ppm integrating to two protons each, matched to proton pairs 5,6 and 2,7 accordingly. In the ^{13}C spectrum a total of fourteen signals were recorded, with the carbonyl carbon located the most downfield at 177.05 ppm. The high-resolution mass spectrum recorded a signal at 240.0672, matching the calculated $[\text{M}+\text{H}]^+$ ion, 240.0661.



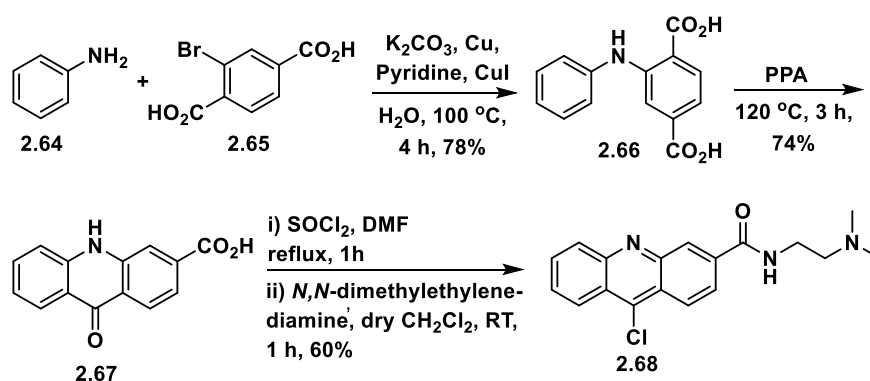
Scheme 2.4: Synthesis of 9-chloroacridine-4-carboxamide (**2.63**) by conversion of acridone-4-carboxylic acid (**2.62**) into the acid chloride, followed by amination with *N,N*-dimethylethylenediamine

To form 9-chloroacridine-4-carboxamide (**2.63**), acridone-4-carboxylic acid (**2.62**) was converted to an acid chloride, by refluxing in thionyl chloride with a catalytic amount of DMF for one hour, also substituting the ketone with a chloride, following a procedure published by Denny.³⁵¹ Upon completion, the thionyl chloride was removed *in vacuo* and the product was used for the next step without any purification. An excess of *N,N*-dimethylethylenediamine in anhydrous dichloromethane led to the amidation of the acid chloride, yielding the 9-chloroacridine-4-carboxamide. The product was purified via column chromatography yielding a yellow oil which was finally triturated with hexane to afford a yellow solid. The final product was synthesised with a 64% yield.

The addition of the carboxamide side chain onto **2.63** was confirmed by ^1H NMR, the presence of a sharp singlet at 2.45 ppm integrating to six protons, corresponding to the two methyl groups attached to the tertiary amine, not coupled to any protons. Additionally, the triplet at 3.78 ppm and the triplet at 2.72 ppm can be assigned to the methylene group protons, a and b, accordingly, coupled to each other. The addition of the side-chain was further confirmed from the ^{13}C spectrum, with the addition of three signals located upfield corresponding to the a, b carbons and two methyl carbons. The high-resolution mass spectrum recorded a signal at 328.1217, matching the calculated $[\text{M}+\text{H}]^+$ ion, 328.1137.

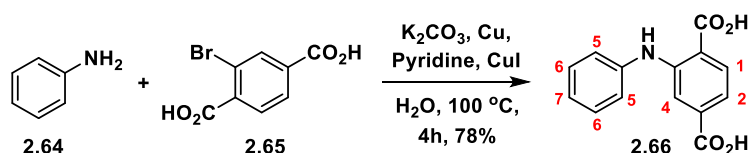
2.3.2 Synthesis of 9-chloroacridine-3-carboxamide monomer (2.68)

Compound **2.68** was successfully synthesised through a series of three steps shown in **Scheme 2.5**, similar to the synthetic pathway described in **Scheme 2.1**. The starting dibenzoic acid, (phenylamino)terephthalic acid (**2.66**), was prepared following a modified Ulmann-Goldberg reaction developed by Ferguson.³⁵³



Scheme 2.5: Synthetic pathway for 9-aminoacridine-3-carboxamide

The reaction occurs between 2-bromoterephthalic acid (**2.65**) and aniline (**2.64**), with copper, copper iodide and pyridine acting as co-catalysts, the reaction is presented below, **Scheme 2.6**. The reaction shares similarities to the Jourdan-Ulmann reaction used to prepare compound **2.61** in **Scheme 2.2**.

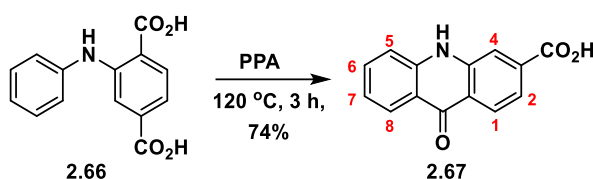


Scheme 2.6: Modified Ulmann-Goldberg reaction between aniline and 2-bromoterephthalic acid with co-catalysts copper, copper iodide and pyridine

Pure product was isolated following a celite filtration and an acid-base workup. Finally, recrystallisation from EtOH/H₂O gave the product as a yellow solid in a 78% yield.

The ^1H NMR spectrum of **2.66** showed a broad singlet at 9.60 ppm corresponding to the NH proton, confirming the coupling of the starting materials. A total of five signals were found in the aromatic region, in line with the eight protons. The furthest downfield signal is a doublet integrating to one proton, corresponding to proton 1, due to its proximity to the carbonyl group and its *ortho*-coupling to proton 2. A doublet located at 7.73 ppm can be matched to proton 4, with a small coupling constant of 1.4 Hz to proton 2, also located at the alpha-position of the carbonyl group. The remaining signals are three multiplets at 7.38-7.44, 7.26-7.30 and 7.12-7.17 ppm. The multiplets integrate to two, three and one proton accordingly, and correspond to protons 6, 5+2 and proton 7, assigned through the analysis of a COSY NMR experiment. The high-resolution mass spectrum calculated for compound **2.66** was found to be 258.0766, matching the calculated $[\text{M}+\text{H}]^+$ ion 258.0688.

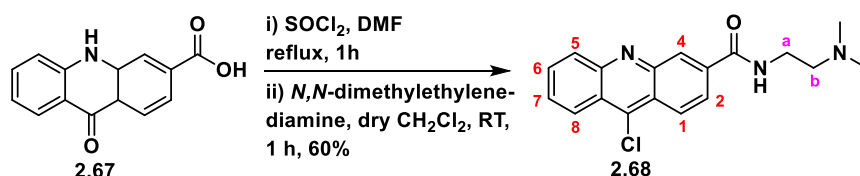
To synthesise the acridone-3-carboxylic acid, compound **2.66** was heated under reflux in polyphosphoric acid (PPA) to complete the cyclisation step, using a procedure described by Bretonnier.³⁵⁴



Scheme 2.7: Synthesis of acridone-3-carboxylic acid (**2.67**) with polyphosphoric acid

Following an acid-base workup, the acridone-3-carboxylic acid (**2.67**) was isolated as pure product in a 74% yield.

The ^1H NMR spectrum is similar to that of compound **2.66** with the total number signals found in the aromatic region equating to a total of seven protons, instead of eight. Two doublets at 8.30 and 8.24, followed by a singlet at 8.18 were matched to protons 1, 8 and 4 respectively, located downfield due to their proximity to the ketone (protons 1 and 8) and the NH as well as the carbonyl (proton 4). The high-resolution mass spectrum was recorded at 240.0610, agreeing with the calculated $[\text{M}+\text{H}]^+$ ion, 240.0582.



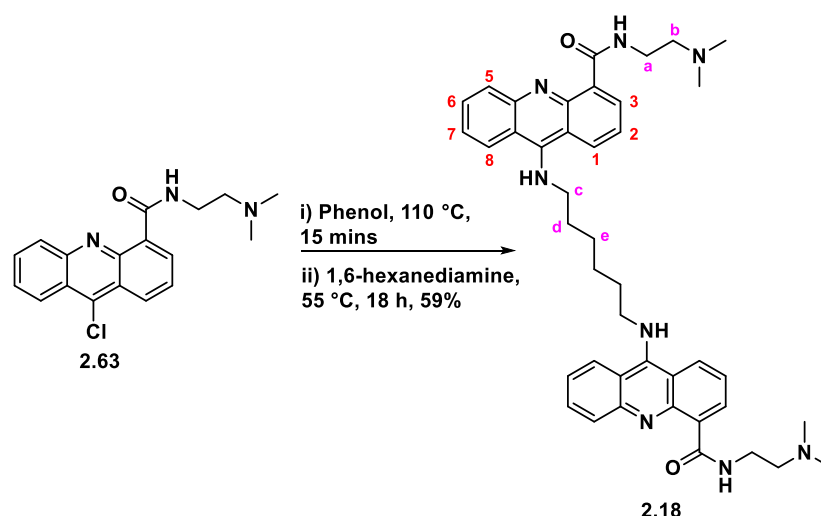
Scheme 2.8: Synthesis of 3-carboxamide-9-chloroacridine by conversion of acridone-3-carboxylic acid into the acid chloride, followed by amination with *N,N*-dimethylethylenediamine

The final step to prepare 9-chloroacridine-3-carboxamide (**2.68**), summarised in **Scheme 2.8**, consists of the same steps followed when synthesising its regioisomer, compound **2.63**, as discussed earlier in **Scheme 2.4**. Pure product was isolated with a 60% yield.

The presence of a sharp singlet 2.37 ppm in the ^1H NMR, corresponding to the methyl protons bound to the tertiary amine located on the added side chain verifies the formation of the desired product. The triplet at 3.65 ppm and the triplet at 2.37 ppm correspond to the rest of the side chain signals, protons located at carbons a and b accordingly, only coupled to each other. The high-resolution mass spectrum calculated for compound **2.68** was found to be 327.1227, corresponding to the $[\text{M}+\text{H}]^+$ ion 327.1217.

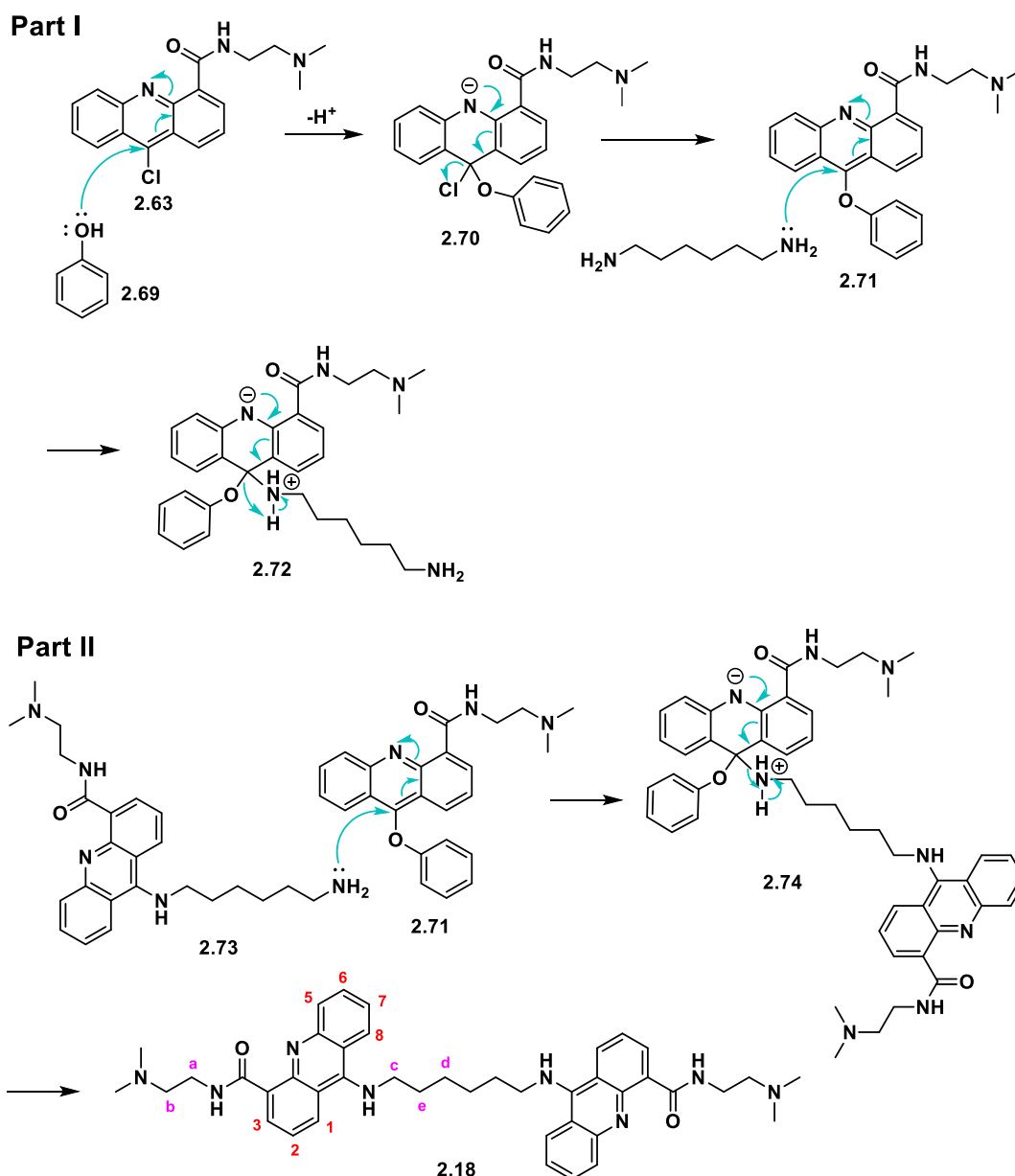
2.3.3 Synthesis of six-carbon linked dimers (**2.18**, **2.75**)

Once the acridine monomers had been successfully synthesised, a procedure published by Wakelin and co-workers was followed to prepare acridine dimers with a selection of linkers.³¹⁷ The first linker chosen was a six-carbon chain, to synthesise previously discussed compound **2.18**, known to bind to four-way junctions.



Scheme 2.9: Synthesis of 9-aminoacridine-4-carboxamide dimer **2.18**, bearing a six-carbon chain linker

Starting material **2.63** was stirred in excess phenol at 110 °C for 15 minutes, resulting in the formation of the phenoxy intermediate (**2.71**), stabilized by the electron-withdrawing effect of the acridine nitrogen, as seen **Scheme 2.10**. This reaction undergoes a $\text{S}_{\text{N}}\text{Ar}$ mechanism, therefore, a Meisenheimer complex is formed. The temperature is reduced to 55 °C, followed by the addition of 1,6-hexanediamine in a 2.1:1 ratio of compound **2.63** to linker, which was allowed to react overnight.



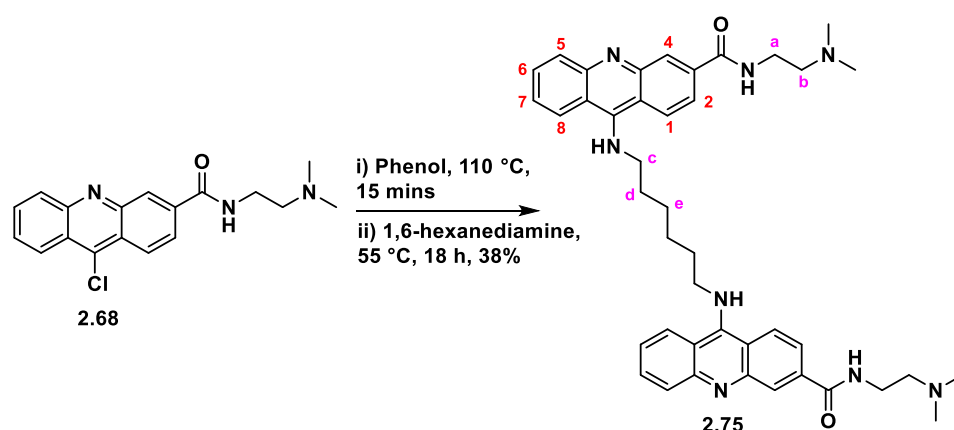
Scheme 2.10: Heating of the **2.63** with phenol leads to the formation of a phenoxy intermediate (**2.71**). Addition of the diamine leads to the formation of the dimer with a S_NAr reaction occurring on each side

Upon completion of the reaction the product was isolated via a basic workup followed by column chromatography. Due to the basic nature of the compound, 1% triethylamine was used in the mobile phase. The obtained bright yellow oil was stirred in a 1.25 M HCl solution in methanol, converting it to tetrahydrochloride salt and precipitated with ethyl acetate, to give **2.18** as a bright yellow solid, with a 59% yield.

When looking at the aromatic region of the recorded ^1H NMR spectrum for **2.18**, there are six signals integrating to a total of twenty eight protons, due to the symmetry of the compound. The addition of the linker was confirmed by the presence of a triplet at 4.15 ppm ($J = 7.3$ Hz), corresponding to protons located at carbon c. The downfield shift is observed due to their proximity to the secondary amine, which is deshielding

the protons, due to conjugation of the nitrogen lone pair with the aromatic system. Additionally, two multiplets at 2.04 and 1.58 ppm correspond to protons, d and e respectively. The HRMS for compound **2.18** was found to be 699.4148, corresponding to the $[M+H]^+$ ion calculated 699.4135.

To examine the contribution of the carboxamide chain's position in DNA binding, analogues were prepared with the chain on carbon 4 for both acridine moieties and on carbon 3. Subsequently, when comparing them in biophysical assays the only factor attributing to any variations would be the position of the threading side chain. The 9-aminoacridine-3-carboxamide **2.75** dimer was prepared in an identical fashion to its regioisomer compound **2.18**. After a basic workup followed by column chromatography, the dimer was converted to the tetrahydrochloride salt to give a bright yellow solid in a 38% yield.

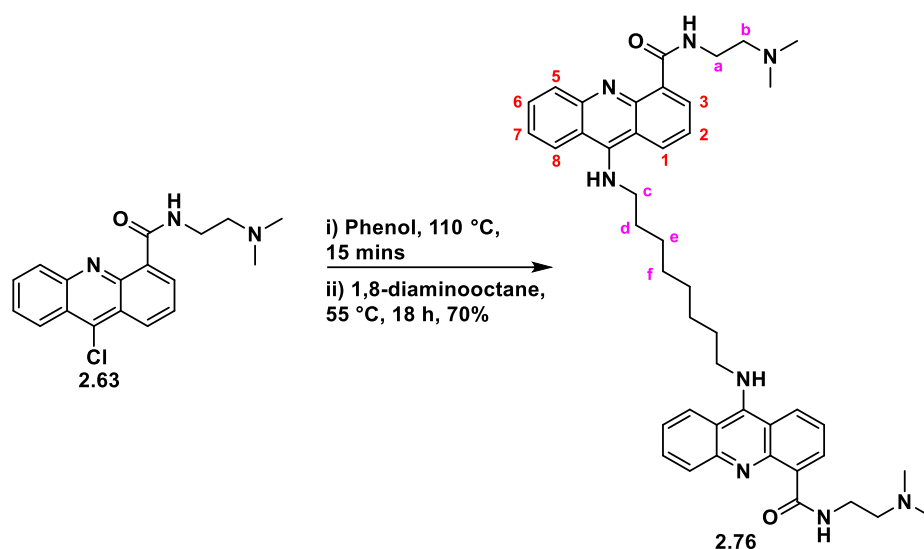


Scheme 2.11: Synthesis of 9-aminoacridine-3-carboxamide dimer **2.75**, bearing a six-carbon linker

In the ^1H NMR spectrum of **2.75**, three extra signals are located in the aliphatic region compared to **2.68**, corresponding to linker protons. A triplet at 4.17 ppm corresponds to methylene group c, adjacent to the secondary amine. Two multiplets at 2.06 ppm and 1.63 ppm correspond to the remaining methylene groups d and e, further away from NH group. The low-resolution mass spectrum confirmed the mass 699.3, matching the calculated $[M+H]^+$ ion 699.4.

2.3.4 Synthesis of eight-carbon linked dimers (**2.76**, **2.77**)

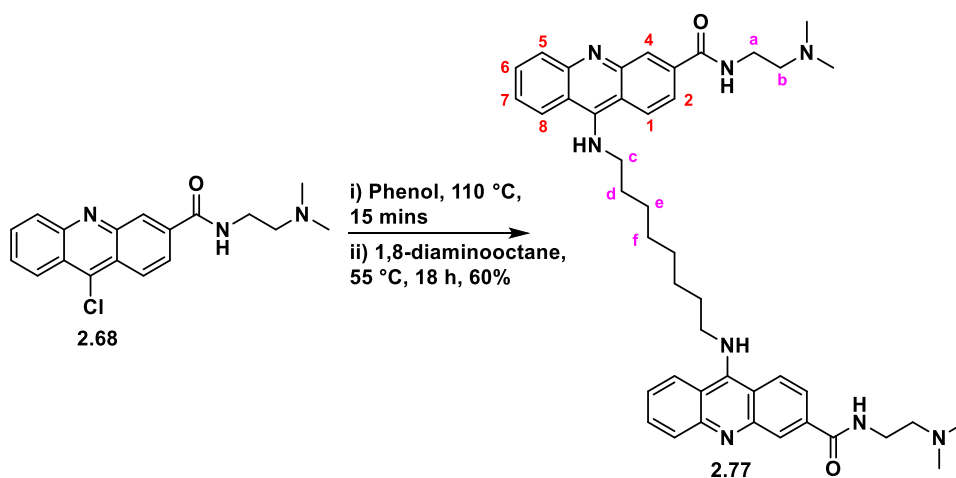
The next pair of acridine dimers synthesised included an eight-carbon chain linker. The 9-aminoacridine-4-carboxamide dimer **2.76**, has been shown to have the ability to crosslink two DNA molecules, as described earlier in the chapter. Therefore, it will act as a control when assessing the rest of the dimers' abilities in carrying out duplex-to-duplex DNA crosslinking.



Scheme 2.12: Synthesis of 9-aminoacridine-4-carboxamide dimer **2.76**, bearing an eight-carbon linker

The acridine monomer **2.63**, was heated in phenol for 15 minutes to form the phenoxy intermediate, which was then reacted with 1,8-diaminooctane to form the desired eight carbon linked acridine dimer. The compound was isolated by a basic workup followed by column chromatography in basic conditions. The product was converted into the tetrahydrochloride salt, yielding a bright yellow solid, isolated with a 70% yield.

The ^1H NMR spectrum of **2.76** (**Scheme 2.12**) was very similar to that of compound **2.18**, the only difference being the multiplet at 1.38-1.54 ppm. The signal integrates to a total of eight protons, as it corresponds to the protons found on carbons e and f, which overlap due to their similar proximity to the two secondary amines located at each end of the linker. Further verification of the structure was given by high resolution mass spectrometry, where the mass was calculated to be 727.4453, corresponding to the $[\text{M}+\text{H}]^+$ ion, 727.4448.



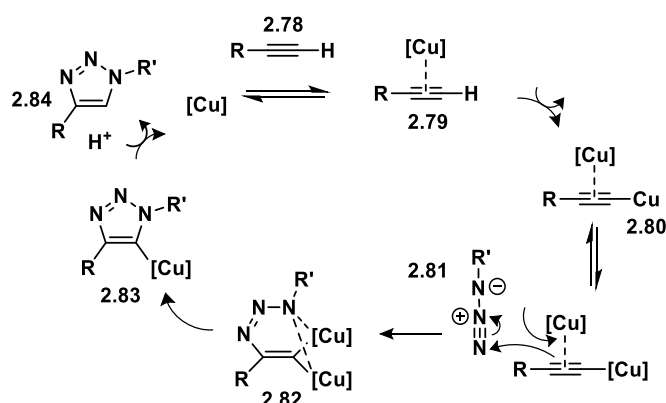
Scheme 2.13: Synthesis of 9-aminoacridine-3-carboxamide dimer **2.77** with an eight-carbon chain linker

Following the procedure described in **Scheme 2.12**, the regioisomer dimer **2.77** was synthesised. To our knowledge this compound has not been described before. Upon conversion to the tetrahydrochloride salt, the compound was prepared with a 60% yield.

The ^1H NMR spectrum of **2.77** holds large similarities to that of compound **2.75** as the structures are near identical. The signal allowing us to confirm the formation of the product is located the furthest upfield at 1.43 – 1.56 ppm, a multiplet integrating to eight protons in total, corresponding to protons located at carbons e and f, due to their proximity to any electronegative atoms. Product formation was further verified through low-resolution mass spectrometry, where the m/z was calculated at 727.4, matching the value for the $[\text{M}+\text{H}]^+$ ion, 727.4.

2.3.5 Synthesis of triazole linked dimers (2.87, 2.94)

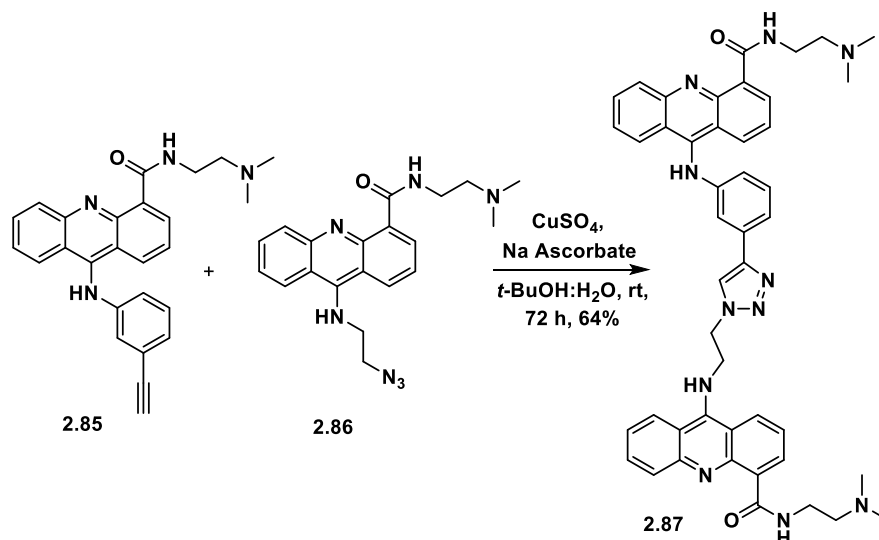
The acridine dimers discussed so far contain carbon-atom only linkers, allowing the compounds enough flexibility to potentially bisintercalate into one double-stranded DNA molecule. However, the strategy chosen to selectively target 4WJs over dsDNA entails preventing the dimers from binding in this mode, forcing them to stretch over the junction and bind to two arms simultaneously. To accomplish this, the flexibility of the linkers needs to be restricted, which can be achieved by introducing a triazole group through “click” chemistry.³²⁰ The triazole was synthesised through a Huisgen’s 1,3-dipolar cycloaddition (CuAAC)) reaction between an alkyne and an azide. This is a Cu(I) catalysed reaction, whose proposed mechanism is shown in **Scheme 2.14**.³⁵⁵



Scheme 2.14: Proposed mechanism of copper(I)-catalysed azide-alkyne cycloaddition, by Fokin et al.³⁵⁵

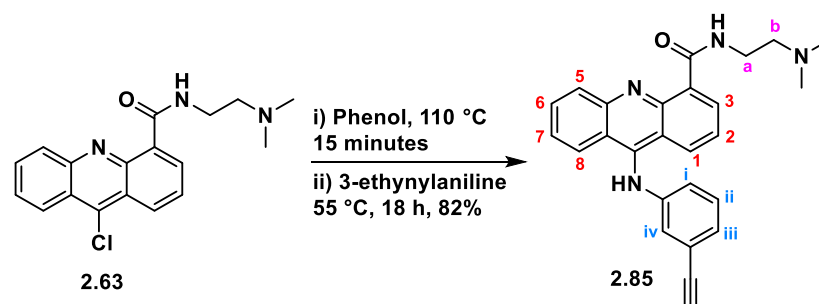
To form dimeric compounds, the alkyne-azide click partners were first synthesised, following procedures published in literature.³²⁰ The alkyne acridine (**2.85**) was designed with the addition of a phenylamino group and the azide “click” partner (**2.86**)

was linked to the acridine via a small carbon linker, retaining some flexibility in the dimer to allow for adjustment upon binding.



Scheme 2.15: “Click” reaction followed for the formation of a 9-aminoacridine-4-carboxamide dimer **2.87**

Since the alkyne group was commercially available, compound **2.85** was synthesised following the same conditions described in **Scheme 2.9**, for dimer preparation.



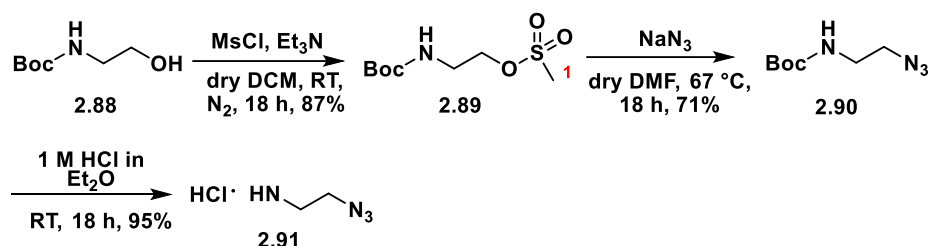
Scheme 2.16: Coupling of (**2.63**) with 3-ethynylaniline, forming the alkyne click partner

Chloro-acridine **2.63** was heated in phenol at 110 °C for 15 minutes to form the phenoxy intermediate **2.71**. The temperature was reduced to 55 °C followed by the addition of one equivalent of 3-ethynylaniline, which was allowed to stir for five hours. The compound was purified by a basic workup followed by column chromatography and finally turned into a tetrahydrochloride salt, with an 82% yield.

In the ^1H NMR spectrum of **2.85**, a total of five signals were present in the aromatic region, integrating to a total of eleven protons. Protons located at positions 1, 3 and 8 are found the furthest downfield, in order, due to their proximity to the amino group at position 9 as well as the carboxamide group on position 4. The signals corresponding to protons 5 and 6 are overlapped and located next. The furthest upfield aromatic signal is a broad multiplet at 7.13 – 7.54 ppm integrating to six protons, corresponding to the remaining acridine signals 2 and 7 as well as the four

new phenyl protons i – iv. The aliphatic region remains similar to **2.63**, with the addition of a singlet at 3.56 ppm, confirming the presence of the alkyne group, whose proton is not coupled to any other protons. Additionally, the low-resolution mass spectrum obtained showed a signal at 409.0, corresponding to the $[M+H]^+$ ion, 409.1.

A simple two carbon azide linker was chosen for the preparation of the azide-acridine “click” partner. The azide substrate was prepared from commercially available Boc-ethanolamine (**2.88**) following the steps presented below in **Scheme 2.18**.³²⁰

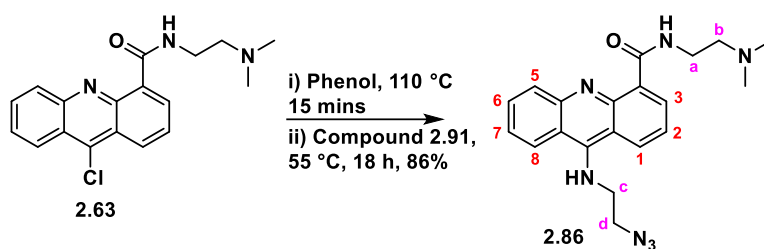


Scheme 2.17: Steps carried out to synthesise 2-azidoethanamine (**2.91**) from Boc-ethanolamine (**2.88**)

The hydroxy group of **2.88** was converted into the mesylate by a dropwise addition of mesyl chloride. Following a workup, the product (**2.89**) was dried to yield pure compound as a yellow oil, at an 87% yield. In the ^1H NMR spectrum of **2.89**, a new singlet, integrating to three protons appeared at 3.02 ppm, corresponding to the methyl protons on carbon 1 of the mesylate group.

Compound **2.89** was heated in anhydrous DMF in the presence of excess sodium azide, resulting in the displacement of the mesylate group by an azide (**2.90**), which was isolated by a workup in a yield of 71%. In the ^1H NMR spectrum, the absence of the singlet at 3.02 ppm confirmed the removal of the mesylate group, additionally a peak measured at 2100 on the IR spectrum, indicative of an azide group, confirmed the addition of the azide group.

Finally, removal of the Boc-protecting group was accomplished under acidic conditions, by stirring compound **2.90** in 1 M HCl in diethyl ether. Upon completion of the reaction, indicated by TLC monitoring, the solvents were removed, and the compound was lyophilized, isolated at a 95% yield. Boc groups can be identified by a characteristic singlet in ^1H NMR around 1.42 ppm, integrating to nine protons, the absence of this peak in the NMR spectrum of **2.91** confirms the Boc group was successfully removed.

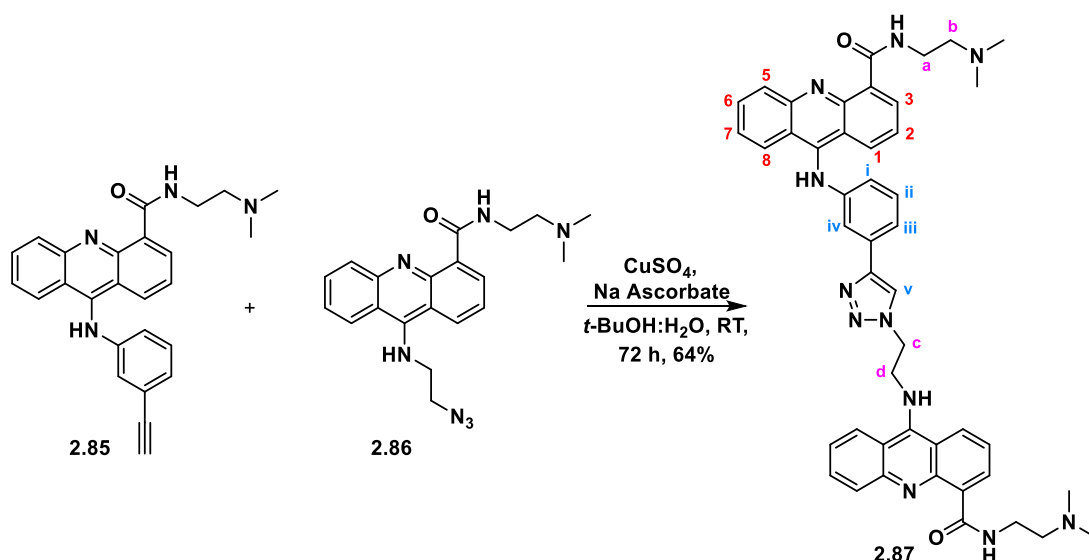


Scheme 2.18: Coupling of the azide substrate to 4-carboxamide-9-chloroacridine to form the azide “click” partner

To complete the synthesis of the 4-carboxamide azide “click” partner **2.86**, **2.63** was heated in phenol at 110 °C for 15 mins. Upon cooling to 55 °C, 1.2 equivalents of compound **2.91** were added, allowing the reaction to stir overnight. The product was isolated via a basic workup followed by column chromatography as a bright yellow oil with an 86% yield.

The IR spectrum shows a signal at 2098 cm^{-1} confirming the addition of an azide group. Additionally, four triplets are found in the aliphatic region of the ^1H NMR spectrum, integrating to two protons each. These signals correspond to the $-\text{CH}_2$ protons bound to carbons a, b, c and d. LRMS measured a signal at 378.1, matching the calculated $[\text{M} + \text{H}]$ ion at 378.2.

A procedure described in literature by Howell *et al.*³²⁰ was followed for the “click” reaction conditions of the **2.85** and **2.86** “click” partners.



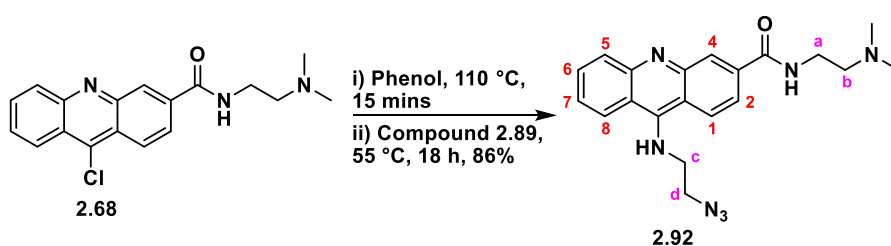
Scheme 2.19: Synthesis of asymmetric 9-aminoacridine-4-carboxamide dimer **2.87** with “click” chemistry

The azide and alkyne acridines were stirred in a 1:1 mixture of *t*-BuOH to water, followed by the addition of sodium ascorbate (10 mol%) and copper sulfate (1 mol%), leaving the reaction to stir at room temperature for 72 hours. Upon completion, the

product was purified via column chromatography, obtaining a bright orange oil, which was converted into the tetrahydrochloride salt, isolated at a 64% yield.

The recorded ^1H NMR spectrum displays a lot of similarities to those recorded for the starting material compounds **2.85** and **2.86**. The aromatic region is made up of eleven signals, integrating to a total of nineteen protons, corresponding to protons on carbons 1 - 3 and 5 - 8 on each acridine group, and the five protons on carbons i-iv. Due to the lack of symmetry of the compound the acridine protons are separate for each side, accounting for fourteen of the aromatic protons. A singlet at 8.13 ppm integrating to one proton, corresponds to the triazole proton on carbon v, confirming the “click” reaction was successful. A total of six triplets are present in the aliphatic region, matching $-\text{CH}_2$ groups c, d and a, b twice, due to the lack of symmetry in the compound, all integrating to two protons. Additionally, two sharp singlets at 2.66 and 2.72 ppm integrating to six protons each, correspond to the methyl protons of the carboxamide chain, confirming it has been unaffected by the reaction. The disappearance of the azide peak in the IR spectrum confirms the azide group had reacted. Lastly, in the high-resolution mass spectrum, a peak was measured at 786.4010, which corresponds to the $[\text{M} + \text{H}]$ ion, 786.3914.

Similarly to the synthesis of the **2.87** dimer, to obtain its regioisomer, the equivalent “click” partners were required. Following the steps in **Scheme 2.18**, compound **2.91** was synthesised and coupled to 3-carboxamide-9-chloroacridine **2.68**.

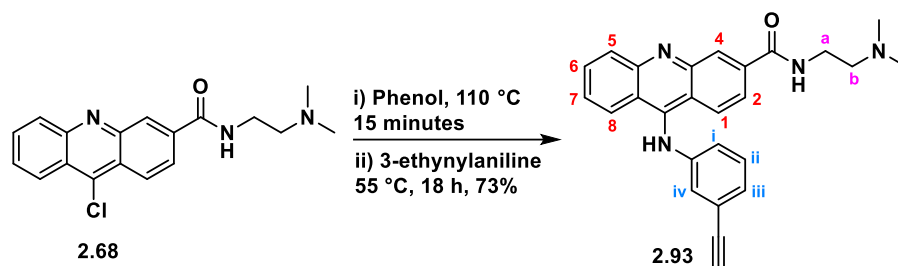


Scheme 2.20: Coupling of azide substrate **2.91** to **3.68** to form the azide “click” partner **2.92**

Compound **2.68** was heated in phenol at 110 °C for 15 minutes. The solution was cooled to 55 °C and compound **2.91** was added, leaving the reaction to stir overnight. The product was purified via a basic workup, followed by column chromatography, isolated at an 86% yield.

In the ^1H NMR spectrum of **2.92**, four triplets are measured in the aliphatic region, each integrating to two protons, corresponding the methylene groups a, b, c and d, confirming the azide chain was successfully coupled. Additionally, from the IR spectrum, a sharp signal was recorded at 2098 cm^{-1} , this indicates the presence of

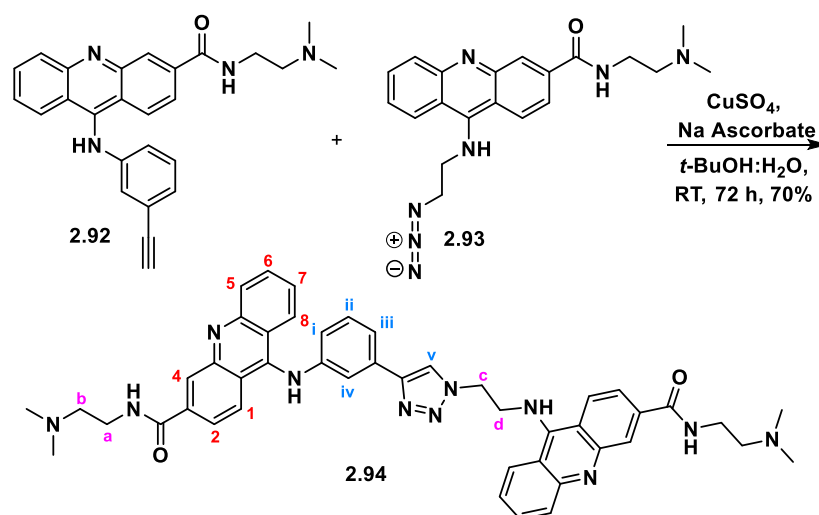
the azide group in the molecule. High-resolution mass spectroscopy measured the $[M+H]^+$ ion 378.2049 at 378.2042.



Scheme 2.21: Coupling of 3-ethynylaniline to **2.68** to form the alkyne “click” partner **2.93**

The alkyne partner was synthesised by heating **2.68** in phenol at 110 °C for 15 minutes, followed by the addition of 3-ethynylaniline once cooled to 55 °C and left to stir overnight. Upon completion, a basic workup and column chromatography were carried out to purify the compound, isolated at a 73% yield.

The ^1H NMR spectrum showed a total of five signals integrating to a total of eleven protons confirming the presence of the new benzene protons i-iv. In the aliphatic region, the carboxamide chain signals remain, with the addition of a sharp singlet at 3.66 ppm corresponding to the alkyne proton, further confirming the structure of the compound. High-resolution mass spectrometry measured 409.2032, matching the $[M+H]^+$ ion 409.1950.



Scheme 2.22: Synthesis of an asymmetric 9-aminoacridine-3-carboxamide dimer **2.94** with “click” chemistry

The two “click” partners **2.92** and **2.93** were coupled to each other in a 1:1 mixture of *t*-BuOH : water, followed by the addition of sodium ascorbate (10 mol%) and copper sulfate (1 mol%), leaving the reaction to stir for 72 hours at room temperature. Purification was carried out by a basic workup followed by column chromatography

and finally conversion into the tetrahydrochloride salt, affording pure product with a 70% yield.

In the ^1H NMR spectrum of **2.94**, ten signals are present in the aromatic region, integrating to a total of nineteen protons. A sharp singlet at 8.41 ppm integrating to one proton corresponds to the triazole proton ν , confirming the reaction was successful. Additionally, four triplets are found in the aliphatic region, two integrating to four protons, corresponding to the a and b protons found on both carboxamide side chains, and two triplets integrating to two protons matched to protons c and d , further confirming the structure of the product. Finally, a signal at 786.4025 was measured in the high-resolution mass spectrum, matching the calculated $[\text{M} + \text{H}]^+$ ion at 786.3914.

2.3.6 Design of rigidly linked acridine dimers

Continuing the synthesis of more rigidly linked dimers, three more pairs were synthesised with varying linkers. To design compounds with the two attached acridines pointing away from each other, benzene and alkyne groups were incorporated into the linkers. Benzene rings are hexagonal planar molecules, and alkynes are linear with a bond angle of 180° , the combination of the two creates rigid linear linkers. The final three linkers designed are shown in **Figure 2.16**.

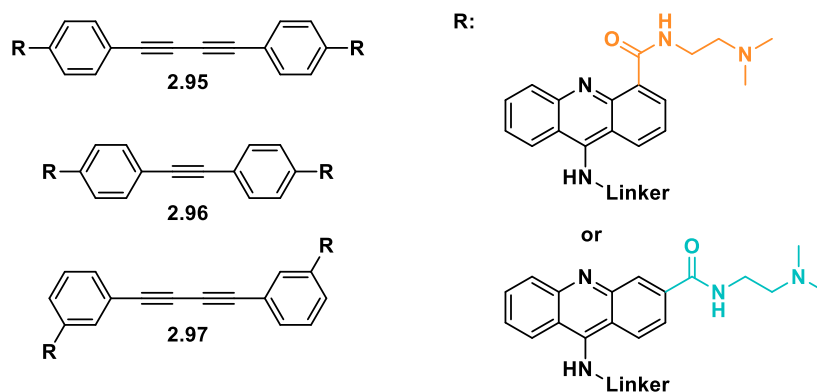


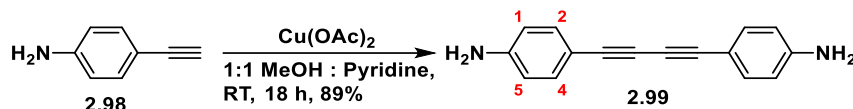
Figure 2.16: Structures of linkers **2.95**, **2.96** and **2.97**, designed to introduce rigidity to 9-aminoacridine-3/4-carboxamide dimers

Linker **2.95** was designed with two benzene rings at each end surrounding two adjacent alkyne groups. Linker **2.96** is similar to **2.95**, however only one alkyne group was used to investigate a shorter linker. Finally, in linker **2.97** the alkyne groups are *meta*-coupled to the acridines, changing the orientation of the dimer. It was important to maintain large similarities between the linkers, as the necessary conformation to allow binding to two arms of a four-way junction, and the distance between the junctions' arms, is unclear. Therefore, by making small changes between the linkers

it would become apparent which features are more important when carrying them through a series of binding assays.

2.3.7 Synthesis of di-4-ethynyldianiline linked dimers (2.100, 2.101)

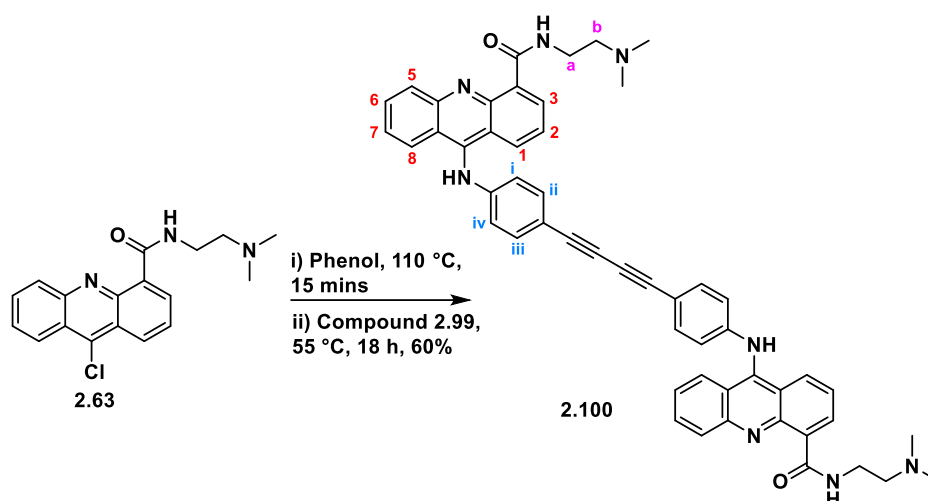
To maintain the dimerisation conditions used in **section 2.3.1**, a diamino version of linker **2.95** was synthesised, following a procedure published by Pachfule *et al.*³⁵⁶



Scheme 2.23: Dimerisation of 4-ethynylaniline (**2.98**) with copper(II) acetate in a MeOH : pyridine solvent mixture

A solution of 4-ethynylaniline was stirred in a 1:1 mixture of MeOH to Pyridine, with copper(II) acetate, at room temperature overnight. Upon completion confirmed by TLC, a workup followed by column chromatography yielded the desired pure compound with an 89% yield.

In the ^1H NMR spectrum of **2.99** there are a total of three signals, two multiplets integrating to four protons each and a broad singlet also integrating to four protons. Due to the symmetry of the compound, protons 1 and 5, and protons 2 and 4, correspond to the same multiplet accordingly. The broad singlet at 5.69 ppm is matched to the four amine protons. A signal was measured in the high-resolution mass spectrum at 233.1082, matching the calculated $[\text{M} + \text{H}]^+$ ion at 233.1079.

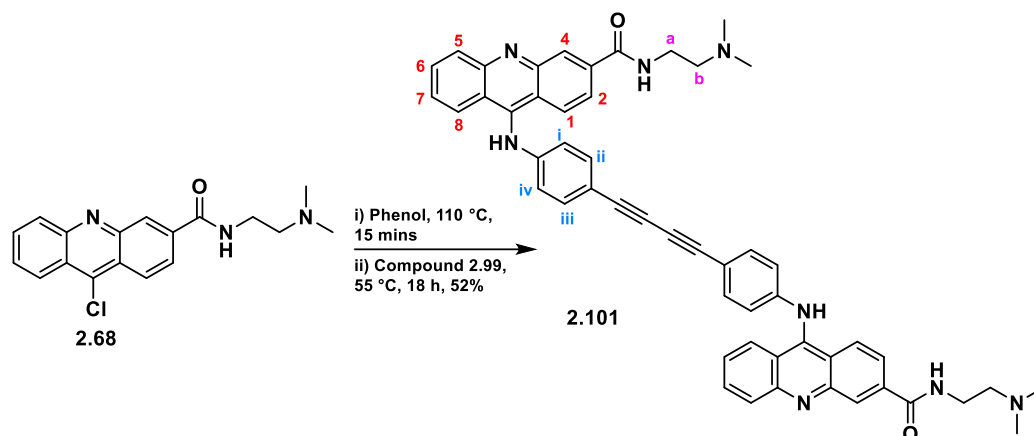


Scheme 2.24: Synthesis of 9-aminoacridine-4-carboxamide dimer **2.100** bearing **2.99** as the linker

Using the previously described dimerisation method (**sections 2.3.1, 2.3.2**) compound **2.100** was synthesis and converted to the tetrahydrochloride salt with a 60% yield.

When analysing the ^1H NMR spectrum of **2.100**, a total of eight signals integrating to twenty two protons are located in the aromatic region. Two new multiplets at 7.69 and 7.46 ppm, integrating to four protons each, coupled to each other, confirm the addition of the linker, corresponding to protons i + iv, and ii + iii. The high-resolution mass spectrum showed a signal at 815.3810, matching the $[\text{M}+\text{H}]^+$ ion 815.3822.

The 3-carboxamide version of compound **2.100** was synthesised in an identical way. The reaction is summarized below in **Scheme 2.25**.



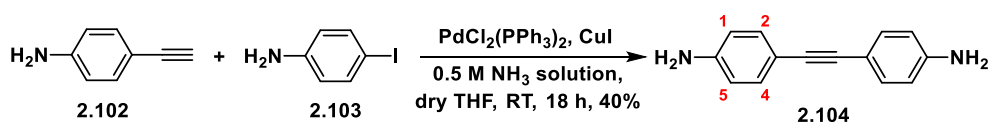
Scheme 2.25: Synthesis of 9-aminoacridine-3-carboxamide dimer **2.101** bearing **2.99** as the linker

The purified product isolated was a bright yellow solid, converted into a tetrahydrochloride salt, synthesized with a 52% yield.

Eight signals integrating to twenty two protons are present in the aromatic region of the ^1H NMR spectrum of **2.101**, confirming the dimerisation. The high-resolution mass spectrum showed a signal at 815.3802, matching the $[\text{M}+\text{H}]^+$ ion at 815.3822.

2.3.8 Synthesis of 4-ethynyldianiline linked dimers (**2.105**, **2.106**)

The next pair of acridine dimers synthesised included linker **2.96**, shown in **Figure 2.16**. The synthesis of the linker was carried out following a procedure described by Pachfule *et al*³⁵⁶. A Sonogashira coupling was performed between an aryl iodide and a terminal alkyne, in the presence of palladium and co-catalyst CuI, generating the conjugated arylalkyne (**2.104**).



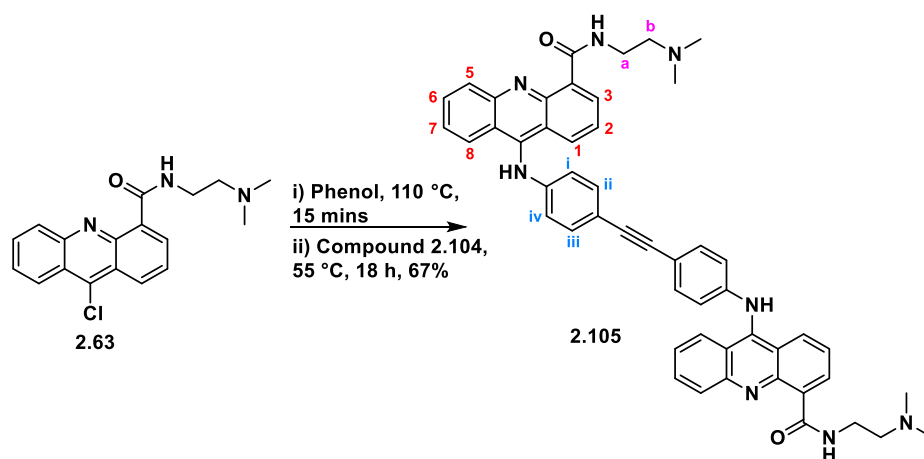
Scheme 2.26: Sonogashira coupling of 4-iodoaniline and 4-ethynylaniline with $\text{PdCl}_2(\text{PPh}_3)_2$ and CuI

In a solution of degassed THF, 4-iodoaniline (**2.103**), $\text{PdCl}_2(\text{PPh}_3)_2$ and copper iodide were added first, followed by the addition of 4-ethynylaniline (**2.102**) and an aqueous

ammonia solution. The reaction was left to stir overnight at room temperature under nitrogen. Upon completion, the solution was extracted with diethyl ether and purified through column chromatography, affording pure product at a 40% yield.

The ^1H NMR spectrum of **2.104** shows a total of three signals. Two multiplets at 7.09 and 6.51 ppm integrate to four protons each, corresponding to the proton pairs 2,4 and 1,5. The more upfield signal is matched to pairs 1,5 due to their proximity to the electron-donating amine. Finally, a broad singlet at 5.40 corresponds to the four amine protons. The high-resolution mass spectrum recorded a signal at 209.1079, matching the calculated $[\text{M}+\text{H}]^+$ ion, 209.1000.

Dimer **2.105** was prepared following the same procedure as **2.100** with the addition of linker **2.104**. The product was isolated and converted into the tetrahydrochloride salt, at a 67% yield.

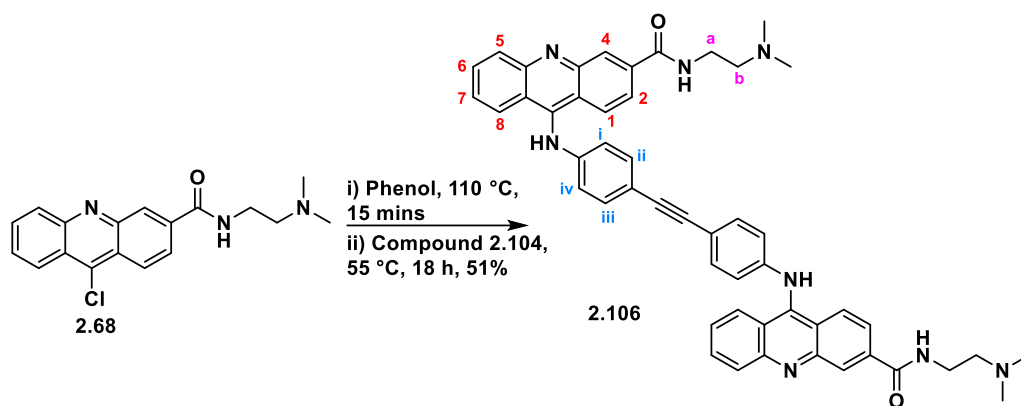


Scheme 2.27: Synthesis of 9-aninoacridine-4-carboxamide dimer **2.105** bearing **2.104** as the linker

Looking at the ^1H NMR spectrum, eight signals are recorded in the aromatic region, integrating to twenty-two protons, corresponding to the acridine and benzene protons. Due to the linkers **2.99** and **2.104** bearing the same number of protons, the ^1H NMR spectrum of compounds **2.100** and **2.105** look near identical. Mass spectroscopy was used to further confirm the desired was formed, with the high-resolution mass spectrum recording a signal at 791.3822, agreeing with the calculated $[\text{M}+\text{H}]^+$ ion, 791.3744.

A 3-carboxamide dimer with compound **2.104** as the linker was synthesised in the same manner as its regioisomer compound **2.105**, outlined in **Scheme 2.29**.

The product was isolated as a bright yellow solid, converted into the tetrahydrochloride salt, with a 51% yield.

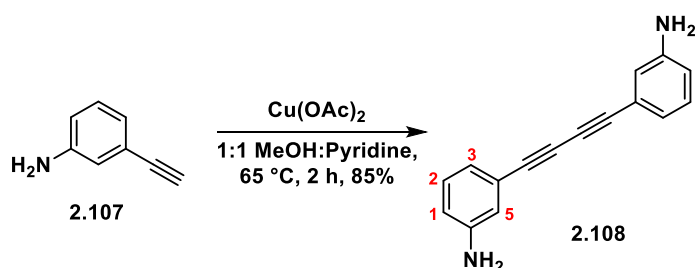


Scheme 2.28: Synthesis of 9-aminoacridine-3-carboxamide dimer **2.106** bearing compound **2.104** as the linker

The ^1H NMR spectrum of **2.106** presented near identical signals to that of **2.105**, with a total of twenty protons in the aromatic region. The high-resolution mass spectrum recorded a signal at 791.3821, matching the $[\text{M}+\text{H}]^+$ ion calculated at 791.3822.

2.3.9 Synthesis of di-3-ethynyldianiline linked dimers (**2.105**, **2.106**)

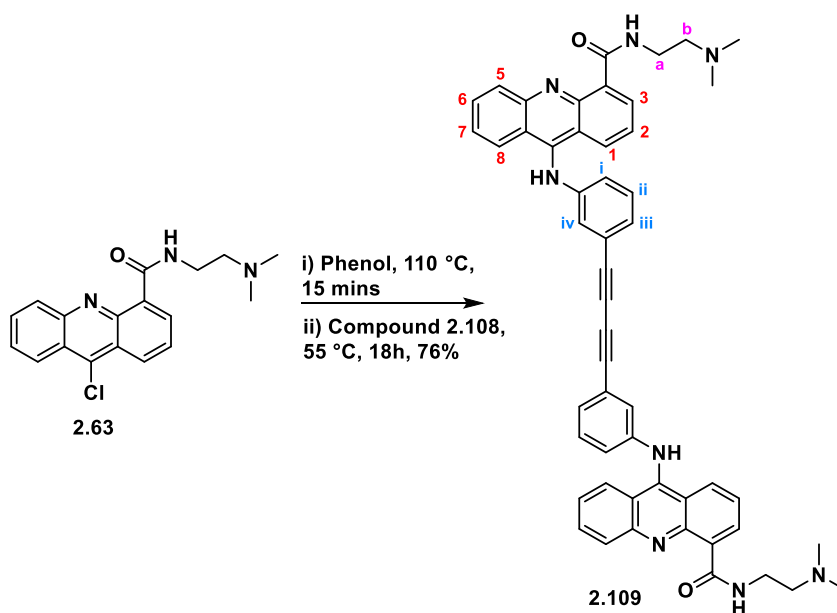
The final linker designed only differs from linker **2.95** in **Figure 2.16**, in the way it is coupled to the acridine monomers. The acridines in compounds **2.100** and **2.105** are *para*-coupled to the aminoacridines, whereas the new linker will be *meta*-coupled. Due to uncertainty of the orientation of compounds **2.100** and **2.105** upon binding to a DNA molecule, it is unclear whether it allows for binding of the second acridine after intercalation. Therefore, by altering the conformation of the compounds with the *meta*-coupled linker and comparing both pairs of acridine dimers, the effect of the linker coupled location can be determined. The linker was synthesised carrying out an Eglinton coupling for the homodimerisation of a terminal alkyne, with a stoichiometric amount of a copper(II) salt in pyridine, to form a symmetrical diyne (**2.108**)^{357,358}.



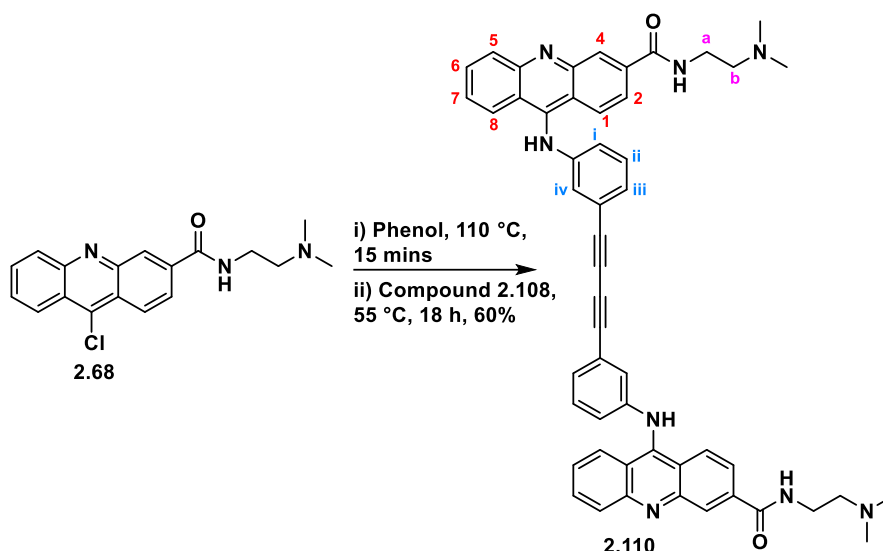
Scheme 2.29: Eglinton coupling of 3-ethynylaniline (**2.107**) with copper(II) acetate in pyridine and MeOH

To synthesise compound **2.108**, 3-ethynylaniline (**2.107**) was heated under reflux in a 1:1 ratio of MeOH to pyridine with copper(II) acetate for 2 hours. The reaction was monitored by TLC. Upon completion, volatile solvents were removed, and the remaining solution was diluted in water. Following an extraction with ethyl acetate the

Compound **2.63** was dimerised with linker **2.108** to afford **2.109**, in a 76% yield. The ¹H NMR spectrum for this compound recorded ten signals in the aromatic region integrating to a total of twenty two protons, accounting for the acridine protons and the linker's aromatic protons. A signal on the high-resolution mass spectrum was recorded at 815.3833, matching the [M+H]⁺ 815.3822.



The final acridine dimer was synthesised in an identical fashion to **2.109**. Dimer **2.110** was synthesised and converted into the tetrahydrochloride salt, with a 60% yield. High-resolution mass spectrometry was carried out, recording 815.3851, matching the $[M+H]^+$ ion 815.3822.



Scheme 2.31: Synthesis of 9-aminoacridine-3-carboxamide dimer **2.110** bearing **2.108** as the linker

2.3.10 Biophysical evaluation of acridine dimers

With the set of compounds in hand (**Figure 2.17**), the focus was on the investigation of the ability of the compounds to bind to 4WJ structures. Initially, the affinity of the compounds for duplex DNA and their ability to crosslink duplex to duplex DNA, which may indicate a mode of binding to 4WJ DNA. These properties were investigated through a fluorescent intercalator displacement assay (FID) and a gold-nanoparticle based crosslinking assay. PAGE experiments were performed, monitoring the ability of the dimers to bind and/or induce the formation of 4WJs, whilst any conformational changes of the 4WJ upon binding of the dimers was measured with a FRET assay. A FRET-based melting experiments investigating stabilisation of duplex, 3WJ and 4WJ DNA in the presence and absence of the dimers, followed by a competition experiment observing structure selectivity. Finally, binding constants for duplex, 3WJ and 4WJ DNA were calculated with a series of fluorescence titration experiments.

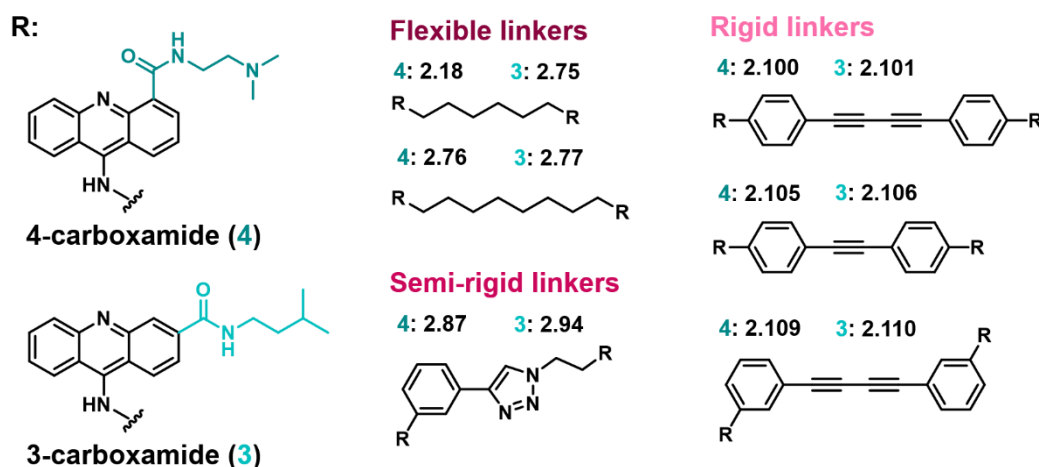


Figure 2.17: Summary of the acridine dimers structures synthesised

2.3.11 Evaluation of duplex DNA binding

A competitive DNA binding assay was performed to determine the binding properties of the synthesised acridine dimers to duplex DNA. One of the strategies designed for junction binding relies on the compounds crosslinking two duplex DNA junction arms simultaneously. Therefore, the binding of these compounds to duplex DNA was investigated through an established binding assay.³⁵⁹ The fluorescent intercalator displacement (FID) assay relies on the competitive displacement of a prebound DNA intercalator, ethidium bromide (EtBr), by the compounds of interest (**Figure 2.18**).^{359,360} EtBr is a well-studied DNA binder, whose fluorescence significantly increases upon binding to DNA, up to 25-fold, compared to its unbound state.³⁶¹ This feature is believed to be the result of the hydrophobic environment surrounding EtBr upon intercalation, leading to the ethidium shedding any water molecules. Water is an effective fluorescence quencher, therefore once distanced from the ethidium, which occurs when it is bound to DNA, the fluorescence increases.³⁶² Equally, if EtBr is displaced, its fluorescence decreases. Thus, this feature can be used to monitor the percentage of EtBr displaced upon addition of a competing compound. The decrease in the measured fluorescence is directly inversely proportional to the extent of DNA binding by the compound and the displacement of the EtBr molecules.

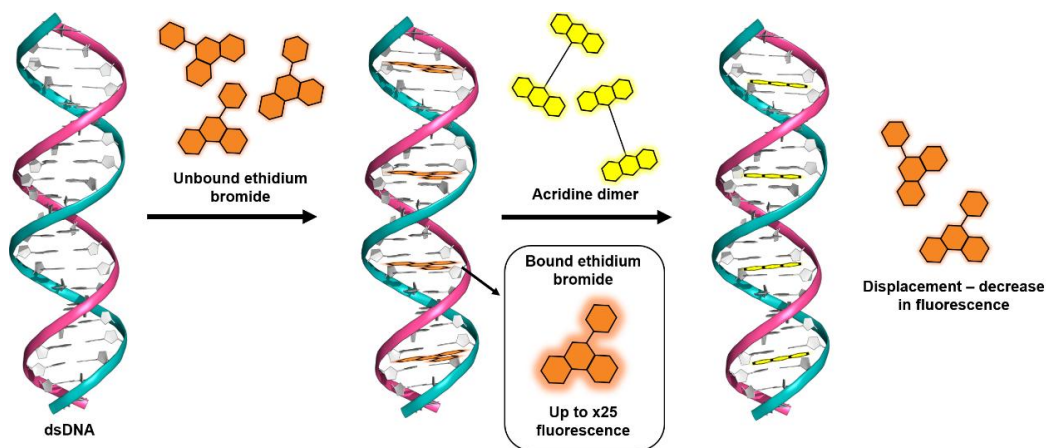


Figure 2.18: Schematic representation of fluorescence indicator displacement assay

This technique was carried out to screen the acridine dimers. However, an important factor had to be taken into consideration, as the acridine dimers are fluorescent themselves at wavelengths 350 – 450 nm. This assay is based on measuring the change in fluorescence of prebound EtBr and the relevant decrease upon addition of a compound, therefore, to measure reliable results there must be no overlap in the absorption wavelengths of EtBr and the acridines. The excitation wavelength used for the monitoring of EtBr is 560 nm. Absorption spectra were obtained for all twelve

compounds between 350 - 600 nm to ensure there was no overlap at 500 – 600 nm. As seen in **Figure 2.19**, there is no interference, as they absorb below 560 nm.

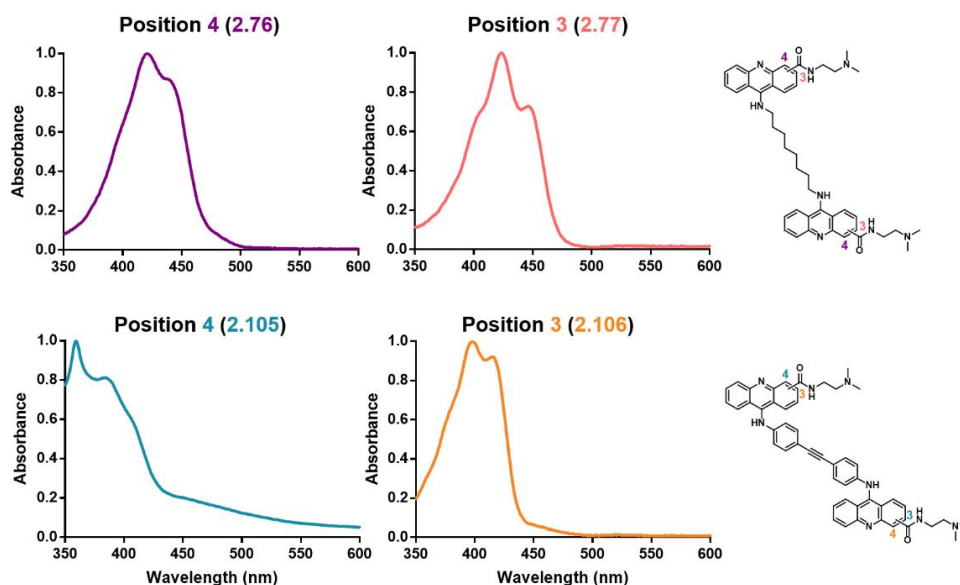


Figure 2.19: UV spectra recorded for compounds **2.76**, **2.77**, **2.105** and **2.106** between 350 - 600 nm

All twelve dimers were tested using this assay, determining DNA binding constants for each compound through the competitive displacement of bound EtBr from the calf-thymus DNA (ctDNA). The compounds were tested at 2 nM up to 200 μ M. Dose-response curves were plotted, calculating a C_{50} value for each acridine dimer. C_{50} was described as the concentration of compound required to displace 50% of the prebound EtBr, observed at 50% reduction in the measured fluorescence.

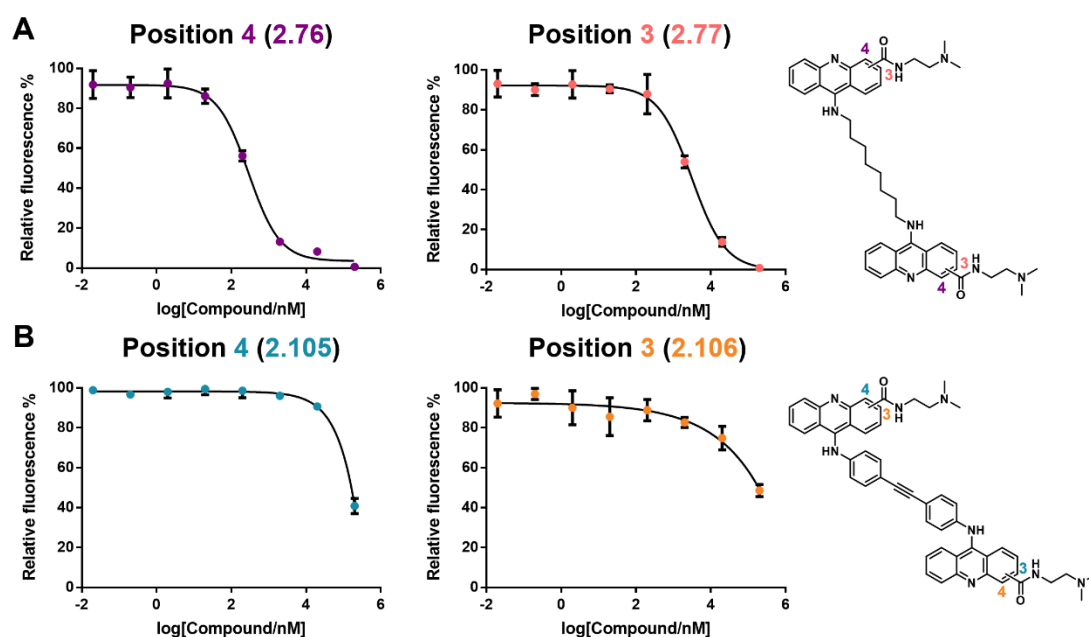


Figure 2.20: Dose-response curves of **A.** compounds **2.76** and **2.77** **B.** compounds **2.105** and **2.106** at ligand concentrations 200 μ M – 2 nM, calf-thymus DNA 8.8 μ M and ethidium bromide 4.4 μ M, in 0.1 M Tris, 0.1 M NaCl at pH 7.0. Graphs were prepared using GraphPad using a dose-response model, $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X) \cdot \text{HillSlope}})$ ($n=3$) ($\pm$ SEM).

9-aminoacridine (9-AA) was used as a control in this assay, measuring a binding constant of $31.910 \pm 2 \mu\text{M}$, agreeing well with literature values reported by Denny and Wakelin.³⁶³ Looking at **Table 2.1** and **Figure 2.20**, six of the dimers showed good DNA binding activity, displacing EtBr at nanomolar concentrations, whereas the other six exhibited poor DNA binding properties. Compound **2.76** was the strongest binder with a C_{50} value of 213 nM, followed by compounds **2.18**, **2.75** and **2.77** in order. The two “click” acridine dimers had DNA binding affinities that were lower than the methylene-linked compounds but higher than the monointercalator 9-aminoacridine. Interestingly, none of the acridine dimers bearing the rigid linkers exhibited good DNA binding. The linear nature of the linkers may cause most of the compound to be directed out of the DNA upon monointercalation, perhaps making unfavourable interactions with the sugar-phosphate backbone and resulting in swift displacing of the dimers. Lack of selectivity due to high dsDNA affinity is one of the major challenges when using small molecules, especially intercalators, to target secondary DNA structures. Therefore, the poor affinity of the rigid dimers for dsDNA is favourable, as it may eliminate dsDNA preference concerns when focusing on four-way junction targeting.

Once the C_{50} values were calculated using the dose-response curves, the apparent binding constants could be calculated as they are inversely proportional to the C_{50} values.³⁶⁴ The following equation was used:³⁶⁵

Equation 2.1: Apparent binding constant equation

$$K_{\text{app}} = K_e \times C_{\text{EtBr}} / C_{50}$$

The concentration of ethidium bromide used was 4.4 μM , and the K_e for ethidium bromide to calf thymus DNA at pH 7.0 and 4.4 μM concentration is $K_e = 9.5 \times 10^6 \text{ M}(\text{bp})^{-1}$.^{365,366} By following this equation for each compound, the apparent binding constants presented in table 1 were calculated.

Table 2.1: Apparent binding constants, C_{50} and LogK values of the acridine dimers tested ($\pm\text{SEM}$).

Compound	C_{50} value (nM)	K_{app} ($\text{M}(\text{bp})^{-1}$)	LogK
2.18 dimer	487 $\pm$ 32	8.58×10^7	7.93
2.75 dimer	655 $\pm$ 65	6.38×10^7	7.80
2.76 dimer	213 $\pm$ 53	19.62×10^7	8.29
2.77 dimer	541 $\pm$ 77	1.64×10^7	7.21
2.87 dimer	3,562 $\pm$ 101	1.74×10^7	7.24
2.94 dimer	6,461 $\pm$ 153	6.47×10^6	6.81

2.100 dimer	>50	Nd	Nd
2.101 dimer	>50	Nd	Nd
2.105 dimer	>50	Nd	Nd
2.106 dimer	>50	Nd	Nd
2.109 dimer	>50	Nd	Nd
2.110dimer	>50	Nd	Nd
9-Aminoacridine	31,910 ± 2,000	1.30 x 10 ⁶	6.11

The logK value calculated for 9-AA of 6.11 compares well to literature values. Wakelin and Denny reported LogK values of 6.08 and 6.11 for 9-AA, when testing against A-T and G-C rich sequences respectively.³⁶³ Due to the poor binding of the six rigidly-linked dimers, no C_{50} , K_{app} or LogK values were calculated.

When comparing the results gathered to literature, the flexibly linked dimers perform similarly to other acridine-base threading DNA intercalators with a highest LogK value of 7.23.³⁶⁷ Here, compound **2.76** exhibits the highest apparent binding constant with a LogK of 8.29, whilst the remaining flexibly-linked dimers have measured values similar to literature. Interestingly, the incorporation of a rigid linker to the acridine dimers largely impacts their duplex DNA binding affinity, suggesting the design of the linkers shows promise in designing acridine dimers selective for DNA junctions over duplex DNA.

2.3.12 Investigation of duple-to-duplex DNA crosslinking

A unique feature of the acridine dimers described earlier is their ability to crosslink two separate DNA molecules at the same time, named duplex-to-duplex DNA crosslinking. This has been confirmed for compounds **2.18** and **2.87** through a gold nanoparticle-based assay, previously developed in collaboration with Dr Marin, summarised in **Figure 2.21**.³⁶⁸

Gold nanoparticles with a 16 nm diameter are characterised by a bright red colour, which changes upon aggregation.^{369,370} This can easily be detected by the naked eye, alongside the change in the UV-Vis spectrum extinction recorded upon small molecule-induced aggregation. AuNPs have surfaces that can be readily modified with various functional groups, such as thiols and amines. These groups can further be used to anchor other molecules to the AuNPs, like ligands, oligonucleotides or proteins. Other groups have demonstrated the use of AuNPs with ssDNA for the purpose of sequence recognition.^{371–373} AuNPs have also been used with dsDNA to determine small molecules' stabilisation effects on duplex DNA.³⁷⁴

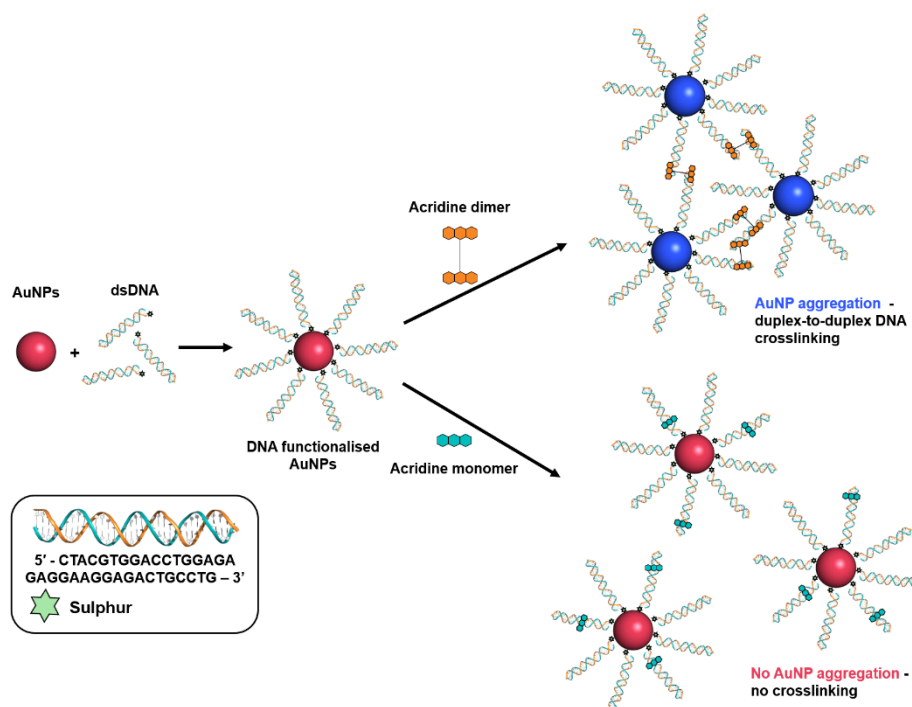


Figure 2.21: Summary of the DNA crosslinking assay upon addition of an acridine dimer (aggregation - blue) and an acridine monomer (no aggregation - red)

The crosslinking assay was used to screen all twelve of the acridine dimers. Gold nanoparticles of 16 nm diameter were synthesised via the citrate reduction method developed by Enüstün and Turkevich.³⁷⁵ The size and shape of the nanoparticles can be controlled with the selected reaction conditions. The size of the synthesised nanoparticles was confirmed with UV-Vis spectroscopy, the peak maxima were measured at 525 nm, which aligns with the expected signal of 16 nm gold nanoparticles. One of the two oligonucleotides was modified with a thiol group at the 5' end to form a gold-sulphur bond for the attachment of the dsDNA molecules to the AuNPs due to its stability. The AuNPs were functionalised with the thiolated dsDNA, following a procedure previously reported by Zhang *et al.*³⁷⁶ Prior to the functionalisation, the DNA was annealed to form the duplex. The known minor preference of acridines for GC base pairs was incorporated into the DNA duplex sequence, allowing for multiple intercalation sites. The functionalised AuNPs hold the characteristic red colour in solution. To test their stability, NaCl was added to the solution, where no effect was seen. NaCl causes aggregation to non-functionalised nanoparticles. It is therefore a convenient method to confirm a functionalisation procedure has been successfully completed.

The acridine dimers were titrated into a PB solution of functionalised nanoparticles to investigate their crosslinking properties. If the acridine dimer can bind to two DNA molecules functionalised onto different nanoparticles, this will cause the AuNPs to be

brought together and form clusters, causing aggregation. There are three signs that confirm aggregation, and by extension DNA crosslinking. Upon formation of the clusters and ultimately aggregation, the absorption spectrum will show an obvious red shift in the peak maxima, as well as a decrease in absorption intensity. Additionally, the red colour of the solution will also disappear.

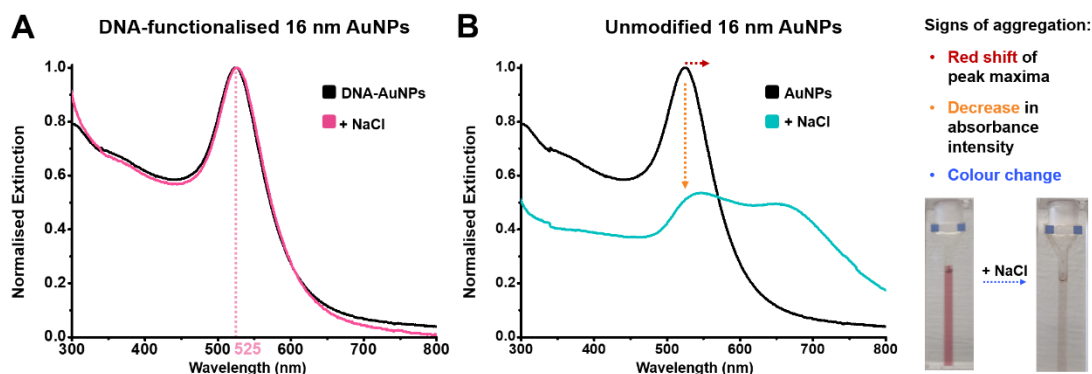


Figure 2.22: **A.** UV spectra of the synthesised 16 nm AuNPs in the absence and presence of NaCl. No change confirms successful functionalisation, **B.** aggregation effect on unmodified AuNP with the addition of NaCl. Spectra was normalised to the absorbance measured at the peak maxima 525 nm.

For each compound, the absorption spectrum of the functionalised AuNP solution was recorded in its absence, and after each titration. A total of ten additions were performed for each compound directly into the cuvette. Between each addition, prior to recording the absorbance spectrum, the sample was shaken by hand to ensure even distribution of the added ligand. Upon completion of a titration, the data was normalised, and an extinction graph was plotted to monitor the changes to the spectra.

Titration of compounds **2.18**, **2.75**, **2.76**, **2.77**, **2.87**, **2.94**, **2.100**, **2.105** and **2.109** led to a red shift in the surface plasmon absorption band, a decrease in intensity and a colour change of the solution. Therefore, these 9 compounds were able to carry out duplex-to-duplex DNA crosslinking at the concentrations investigated, 0 to 17.34 μM . Compounds **2.18** and **2.87** have been disclosed in literature, with the results acquired in this project matching the published data. The acridine dimers bearing flexible linkers **2.18** and **2.76**, were able to crosslink and cause aggregation. This result agrees with the compounds' observed strong DNA binding affinity from the FID assay. Their regioisomers **2.75** and **2.77**, even though they exhibit lower affinity for duplex DNA binding, are still able to crosslink. It is likely the flexibility provided by the linkers allows for the necessary adjustments to improve binding.

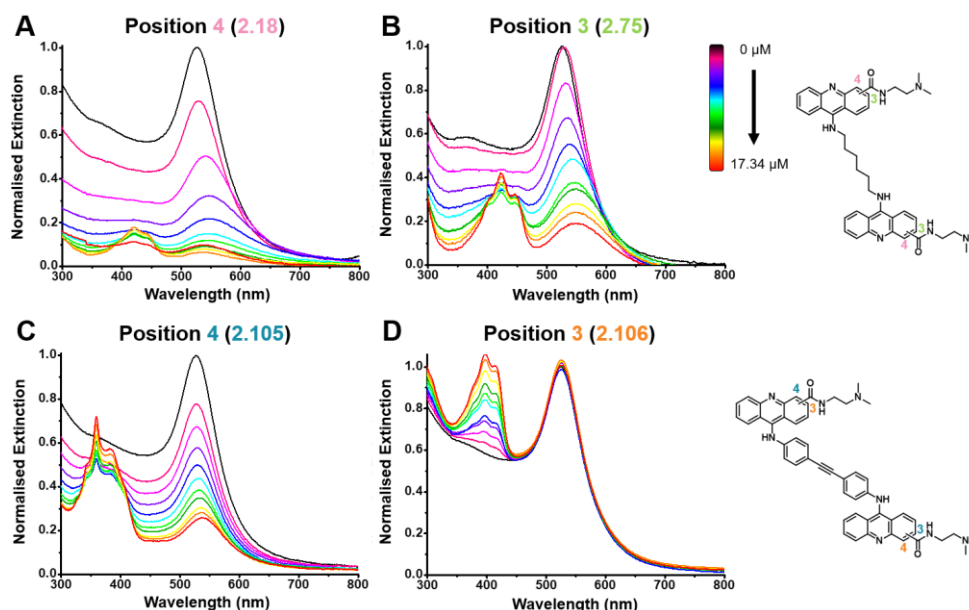


Figure 2.23: UV spectra recorded upon titration of **A.** compound **2.18**, **B.** compound **2.75**, **C.** compound **2.105** and **D.** compound **2.106**, at concentrations 0 - 17.34 μM , to a solution of dsDNA functionalised AuNPs in 10 mM PB, pH 7.4 ($n=3$). The black trace corresponds to the UV trace of the functionalised-AuNPs prior to the addition of any compound.

When compounds **2.100**, **2.105** and **2.109** were titrated into the DNA-functionalised AuNPs, aggregation was observed, due to duplex-to-duplex DNA crosslinking. Since these compounds are poor DNA binders, but perform well in the crosslinking assay, it is likely that simultaneous binding of both acridine groups grants some stability to the complex, making it more favourable than mono-intercalation. This feature could prove useful when targeting 4WJs, ensuring both acridines are bound to the structure and forming a stable DNA-dimer complex.

The remaining three dimers **2.101**, **2.106** and **2.110**, did not show any signs of crosslinking at the chosen concentrations. Shifting the carboxamide chain from position 4 to 3 and replacing the flexible linkers with more rigid ones both heavily influenced the DNA binding properties of the dimers. Therefore, this seemed to prevent the compounds from carrying out duplex-to-duplex DNA crosslinking.

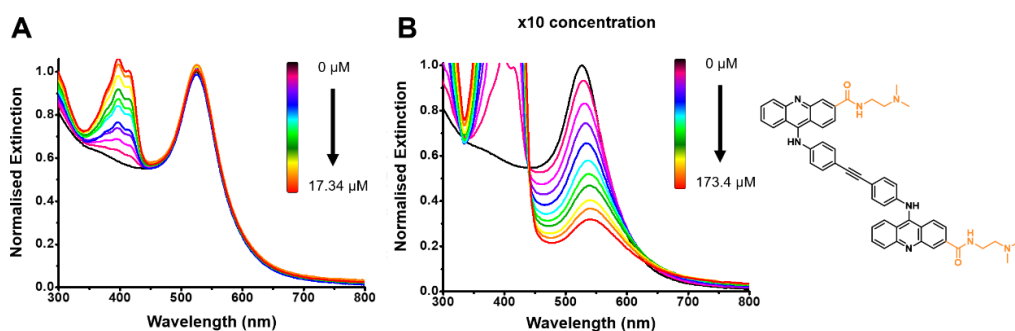


Figure 2.24: UV traces recorded of compound **2.106** at **A.** 0 - 17.34 μM and **B.** 0 - 173.4 μM , to a solution of dsDNA functionalised AuNPs in 10 mM PB, pH 7.4 ($n=3$). The black trace corresponds to the UV trace recorded of the functionalised-AuNPs prior to the addition of compound

However, upon increasing the concentration titrated, they were able to crosslink, leading to AuNP aggregation, **Figure 2.24**. This suggests that the resulting complex is not very stable however a large excess of compound can overcome this issue.

To ensure the change in the solution's colour was not a result of the DNA intercalation, titration of a monomer, 9-aminoacridine, was performed. No change was measured in the UV-Vis spectrum or the solution colour, confirming the observed changes were a consequence of the crosslinking. The assay was also repeated with the titration of PBS, to verify there was no dilution effect. Finally, UV traces were also run of the acridine dimers, to confirm there was no overlap of their absorption wavelengths with the region of interest. The compounds absorb at a lower wavelength than the functionalised nanoparticles, seen in **Figure 2.19**, not interfering with the assay, responsible for the increase in the intensity between 320 – 450 nm observed in **Figure 2.23** and **Figure 2.24**.

2.3.13 Investigation of four-way junction binding by electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (EMSA) allow separation of DNA, RNA and proteins, based on size and charge.³⁷⁷ An electric field is applied to a gel matrix, causing the naturally negatively charged DNA to move from the cathode (-) to the anode (+). Larger DNA fragments encounter higher resistance from the gel matrix, therefore tend to cover less distance compared to smaller fragments, due to their advantage of manoeuvring through the gel pores more easily.³⁷⁸ Different gel consistencies allow for varied separation. Polyacrylamide gels are preferred when handling small nucleic acids, with larger nucleic acids being better suited for agarose gels. Gel electrophoresis experiments have been used to study the geometry of four-way junctions, based on the principle that bent DNA molecules show reduced movement on gels, directly linked to their exact structure and bend angle.^{379,380}

This technique can also be used to study the binding of compounds to 4WJ forming sequences. Four-way junctions are unstable in low divalent cation environments, therefore mostly appear as single stranded bands when visualised on a gel.³⁸⁰ If the addition of a compound stabilises the 4WJ structure, a second band located higher in the gel appears, representing the complex. Under the right conditions, the structure of the DNA can be determined, by separation of the single-stranded DNA and the 4WJ structure (**Figure 2.25**). The 4WJ weighs four times the amount of each oligonucleotide, therefore, travels down the gel at a much slower rate, leading to a clear separation of the bands.

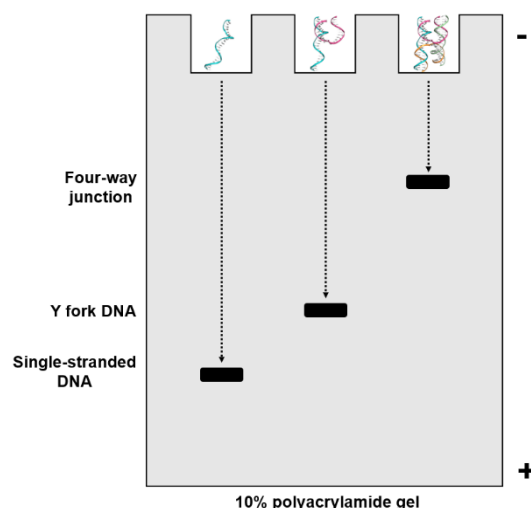


Figure 2.25: Summary of DNA structures travelling down a gel in an EMSA assay

To aid with visualisation of the DNA on the gel, fluorescent tags are often attached to an oligonucleotide. An appropriate filter in the gel imaging system allows for visualisation of DNA assemblies that the labelled oligonucleotide is part of. No fluorophores were used in the experiments described in this thesis, to avoid any unwanted interactions between the tags and the compounds, altering the assay results. Additionally, when working with multiple partially complementary nucleic acids, various secondary structures can be formed, therefore labelling only one strand could be masking other DNA complexes. To avoid this, no labels were attached to the oligonucleotides, and the DNA was visualised using a nucleic acid stain. The most commonly used stain is ethidium bromide, however newer stains have emerged with increased sensitivity. As secondary DNA structures are often unstable, a Sybr Gold nucleic acid stain was chosen.³⁸¹ This is an unsymmetrical cyanine dye shown to have greater sensitivity for detecting nucleic acids in solution and gel electrophoresis. It is characterised by low intrinsic fluorescence with an increase of up to >1000 fold in fluorescence upon binding to nucleic acids. Additionally, its high binding affinity and large signal-to-background ratio make it a preferred tool in nucleic acid staining. When compared to other stains available, it performed the best in detecting both DNA and RNA with high sensitivity, in all gel types tested.³⁸¹

Four partially complementary oligonucleotides were chosen for this experiment, known to form a 4WJ in the appropriate conditions³⁸², previously used in the group and chosen for consistency. Each oligonucleotide has 22 bases, assembling an 88 base-long 4WJ. The sequences are presented below:

X: 5'-GGCAGTTGACGTCATGCTAGGC-3'

R: 5'-GCCACCAACGGCGTCAACTGCC-3'

B: 5'-GCCTAGCATGATACTGCTACCG-3'

H: 5'-CGGTAGCAGTACCGTTGGTGGC-3'

Two types of experiments were carried out, looking into the compound's 4WJ binding abilities under different conditions. The first experiment investigated the stabilisation of the 4WJ in the presence of the compound, whilst the second also assessed the compound's ability to induce the formation of the 4WJ at room temperature.

The four-way junction was prepared by the addition of stoichiometric amounts of the four oligonucleotides, with a final 4WJ concentration of 1.25 μM in TBE buffer. A total of eight concentrations were chosen for each compound following 1:2 dilutions. The compound concentrations were prepared for 1 in 50 dilutions, as 50 μL of the 4WJ mixture were added to each sample with 1 μL of compound. In the stabilisation gels, the solutions were heated to 90 $^{\circ}\text{C}$ and left to cool to room temperature overnight, annealing the DNA in the presence of the compound.

The sequences chosen are not self-complementary, therefore they are expected to exist as ssDNA molecules as they are stored separately. DNA annealing is carried out to encourage base pairing of the complementary sections of the oligonucleotides. During the annealing process, the high temperature ensures any hydrogen bonds between base pairs potentially formed have broken, denaturing the DNA, without altering the DNA backbone³⁸³. The slow cooling that follows allows DNA renaturation, the process in which the hydrogen bonds are reformed after the heat disruption, forming a more favourable and stable complex than ssDNA. Both the denaturation and renaturation steps are affected by the length and the base anatomy of the sequences³⁸⁴. However, renaturation is a much slower process, as it requires the collision of four oligonucleotides to occur in the required complementary order, for all the base pairs to form their hydrogen bonds. This step is performed both in the absence and the presence of various concentrations of the ligand, comparing the amount of 4WJ stabilised. Once the DNA strands have been left to cool overnight, the loading dye was added to each tube. The gel was run for 120 minutes at room temperature, with a constant voltage power of 50 V. Upon completion of the run, the gel was stained with the Sybr Gold nucleic acid stain for 30 minutes before visualisation using an imaging system.

A second gel electrophoresis assay was performed, referred to as 4WJ promotion assay, where the DNA was not annealed. The compound was added to the DNA mixture and left to incubate for 1 hour, to assess whether the compound can induce

the assembly of the 4WJ. The rest of the procedure was carried out the same way, with the addition of the loading dye prior to loading the samples onto the gel.

Compound **2.18** was initially tested, as it is a known 4WJ binding compound and has been explored in gel electrophoresis^{320,385}. Controls were included in all experiments, DMSO was added to one sample with no ligand as a reference for the 4WJ that forms in these conditions naturally (**C1**). A second control well held only the X DNA strand, as an indicator of the ssDNA band's location on the gel (**C2**).

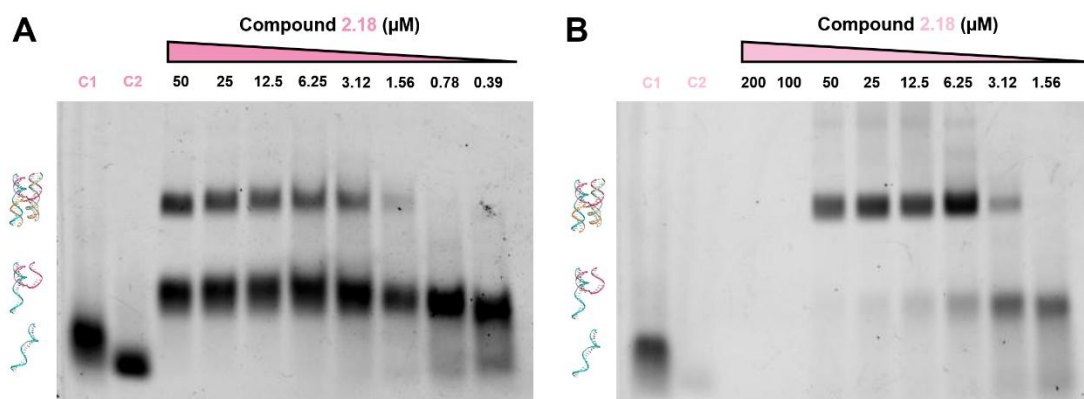


Figure 2.26: Gel images of compound **2.18** in **A**. 4WJ trapping experiment, annealed with X, B, H, R strands overnight, from 50 μM 1:2 dilutions. **B**. 4WJ promotion experiment, incubated with X, B, H, R strands for 1 hour at room temperature, from 200 μM in 1:2 dilutions. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

Compound **2.18** was titrated from 50 to 0.39 μM , producing a 4WJ band between from 50 μM to 1.56 μM in the 4WJ-trapping gel (**Figure 2.26A**). Another band appears above the ssDNA band in the presence of the compound, this is a pseudo-duplex forming a Y fork, potentially stabilised by the compound prior to fully forming the 4WJ.

In the second assay, 4WJ formation is observed at the same concentrations as above, however the Y fork band is considerably less prominent. Additionally, at higher than 50 μM of ligand all bands disappear, this is believed to be the result of DNA condensation. It has been reported that at high concentrations multiple compound **2.18** molecules bind non-specifically to the DNA, forming aggregates³⁸⁵. Interestingly, faint bands appear at the top of the gel, suggesting larger DNA complexes have formed, likely due to crosslinking of secondary DNA structures by the dimer. These gels provide a good starting point for the assessment of the remaining compounds, determining the expected band locations corresponding to ssDNA, Y forks and the HJ when using these oligonucleotides.

Compound **2.76** gave similar results, not reaching 4WJ formation at concentrations as low as compound **2.18** but indicating strong binding. However, less DNA

condensation was observed, as seen in **Figure 2.27**, potentially suggesting less non-specific binding in the 4WJ promotion gel.

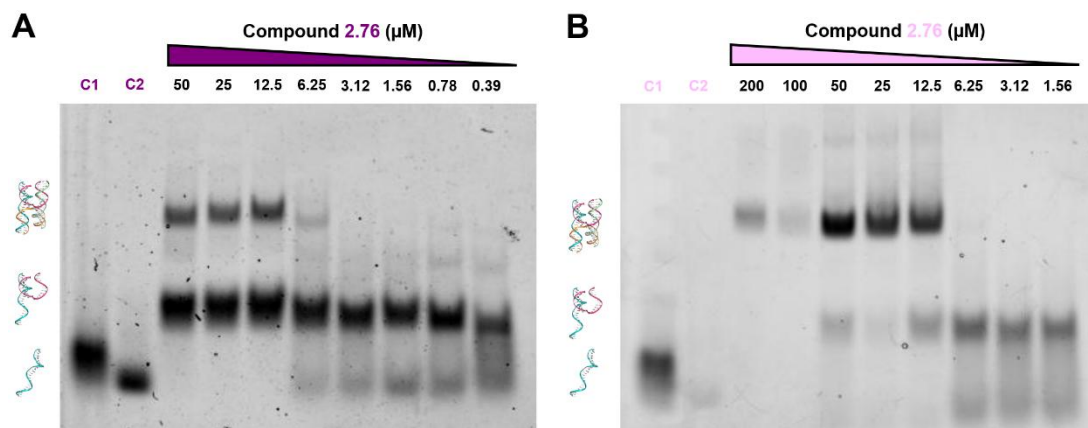


Figure 2.27: Gel images of compound **2.76** in **A**. 4WJ trapping experiment, annealed with X, B, H, R strands overnight, from 50 μM 1:2 dilutions. **B**. 4WJ promotion experiment, incubated with X, B, H, R oligonucleotides for 1 hour at room temperature, from 200 μM in 1:2 dilutions. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

To confirm the middle band did correlate to the pseudo-duplex forks, a control experiments was carried out to pinpoint the location of all the ssDNA molecules and any possible Y fork combinations. Four pairs of Y forks can form, B/X, H/B, R/H and X/R, stoichiometric amounts of the oligonucleotides were annealed overnight. The samples were loaded onto a gel which was subjected to 50 V for 120 minutes. After staining, the image captured after exposure is shown in **Figure 2.28**.

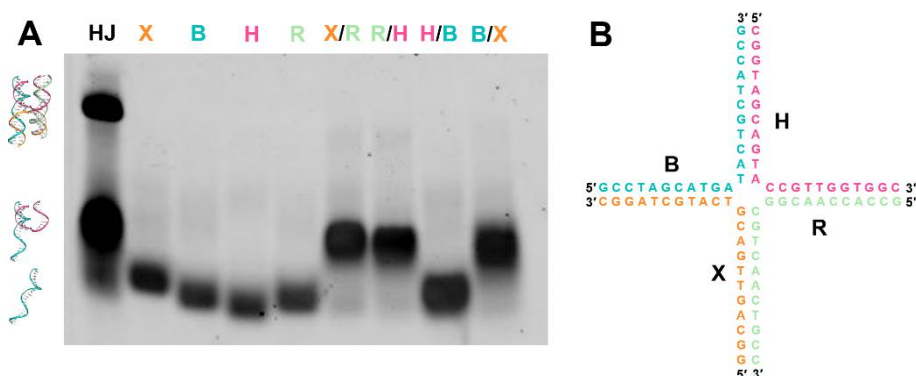


Figure 2.28: **A**. Gel image of X, B, H, R oligonucleotides experiment prepared individually, in pairs X/R, R/H, H/B/ B/X, annealed to form 4WJ. All solutions were prepared to 1.25 μM final DNA concentration in TBE buffer. Gel was ran for 2 hours at 50 V, followed by staining with SYBR Gold stain for 30 minutes prior to imaging. **B**. Representation of the 4WJ structure sequence used in gel electrophoresis experiments.

Compounds **2.75** and **2.77** were analysed in the same manner as their regioisomers, **2.18** and **2.75**, over a range of concentrations to determine whether they can promote the formation of the 4WJ. The highest concentrations chosen was 25 μM , with 1:2 dilutions to 0.19 μM , the gels captures are presented in **Figure 2.29**.

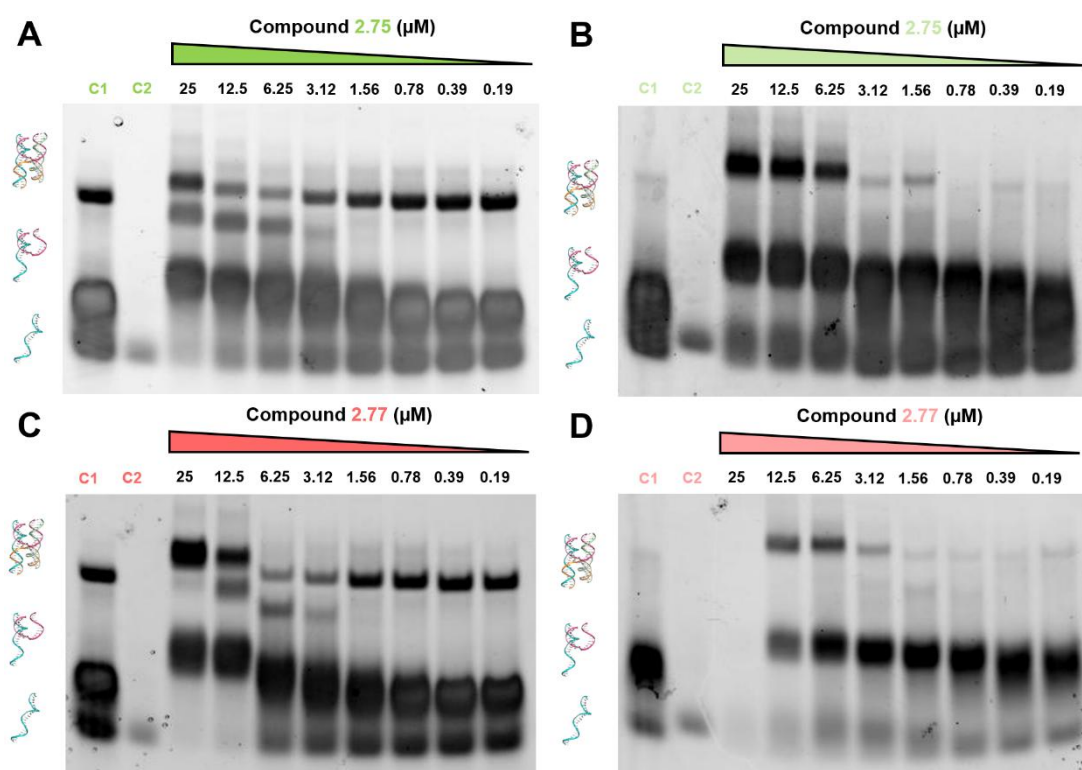


Figure 2.29: Gel images of compounds **2.75** and **2.77** in **A/C**. 4WJ trapping experiments, compound annealed with X, B, H, R strands overnight, from 25 μM 1:2 dilutions. **B/D**. 4WJ promotion experiment, compound incubated with X, B, H, R oligonucleotides for 1 hour at room temperature, from 25 μM 1:2. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

Looking at compound **2.75**, the C1 control in **Figure 2.29A** shows a prominent 4WJ band, binding is observed from 3.12 μM , visible by a slight shift of the 4WJ upon binding. The shift could potentially be associated with a conformation change of the 4WJ occurring upon ligand binding, which can alter how it runs on the gel.²³² At high concentrations, fading of the ssDNA is observed and a fourth band appears right under the 4WJ band. This could be the result of a different 4WJ conformation being formed upon binding of **2.75**, also explaining the fainter top 4WJ bands, as the bend angle of the DNA when assembling a 4WJ affects how it runs on a gel³⁸⁰, no such band appears in the promotion gel. This result suggests that during the incubation, the 4WJ can assemble in the preferred conformation for the most favourable mode of binding by the ligand. This is further supported by the intensity of the 4WJ band, showing high amounts of stabilised 4WJ.

Compound **2.77** holds some similarities to compound **2.75**, as a band below the 4WJ band is shown. However, at 25 μM only two bands are observed, an intense 4WJ band and Y fork band. This suggests that the presence of more compound can shift the equilibrium to the most favourable 4WJ structure. Due to the known dsDNA binding abilities of this compound, measured in the FID assay, it is no surprise that

the rest of the DNA is stabilised in a Y fork shape. When incubated with DNA, a 4WJ band is produced at high concentrations, confirming the compound can induce 4WJ assembly at room temperature, although not as selectively as the 4-carboxamides.

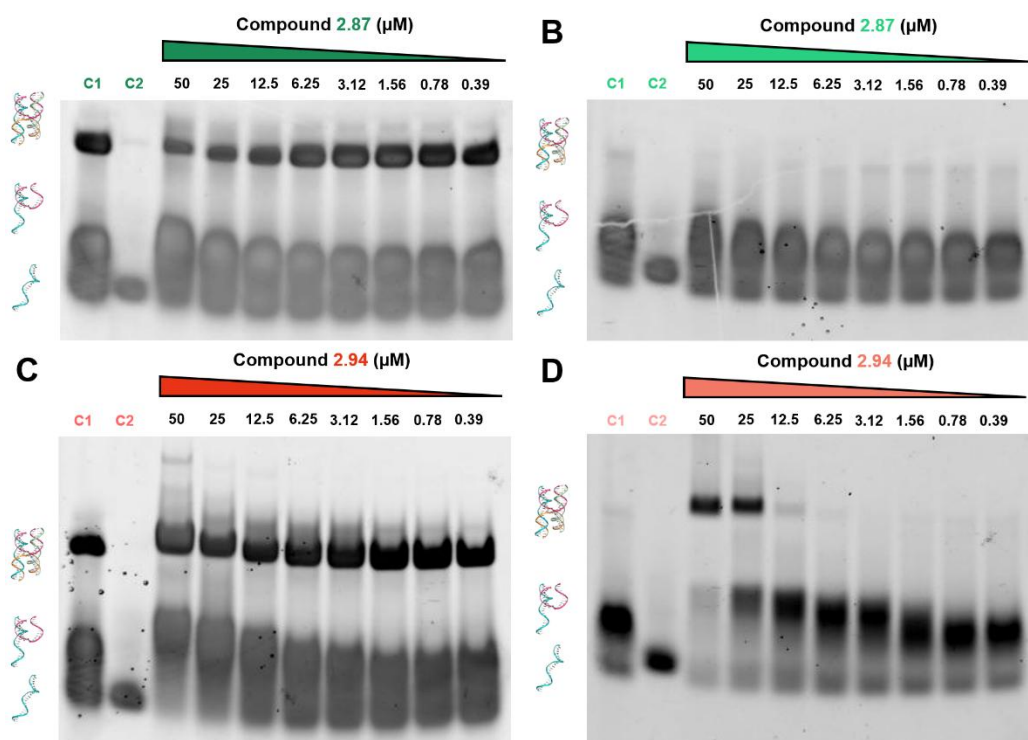


Figure 2.30: Gel images of compounds **2.87** and **2.94** in **A/C**. 4WJ trapping experiments, compound annealed with X, B, H, R strands overnight, from 25 μM 1:2 dilutions. **B/D**. 4WJ promotion experiment, compound incubated with X, B, H, R oligonucleotides for 1 hour at room temperature, from 25 μM 1:2. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

In both 4WJ trapping gels of compound **2.87** and its regioisomer **2.94**, **Figure 2.30A** and **C**, the 4WJ control band is strong, resulting in difficulty when interpreting the effect of the compounds on the gels. A small shift in the 4WJ band is observed in the presence of 50 μM of compound **2.87**, suggesting binding, however there is no clear increase in the intensity of the band. Binding of compound **2.94** to the 4WJ also causes a shift in the 4WJ band, accompanied by an increase in band intensity, suggesting the compound is bound to the 4WJ and stabilising the structure.

The addition of compound **2.87** did not induce formation of the 4WJ at the concentrations tested (**Figure 2.30B**), however, its regioisomer **2.94** does induce 4WJ assembly at concentrations higher than 12.5 μM (**Figure 2.30D**), indicated by a 4WJ band. The appearance of the 4WJ band is accompanied by fading of the ssDNA and Y-fork dsDNA bands.

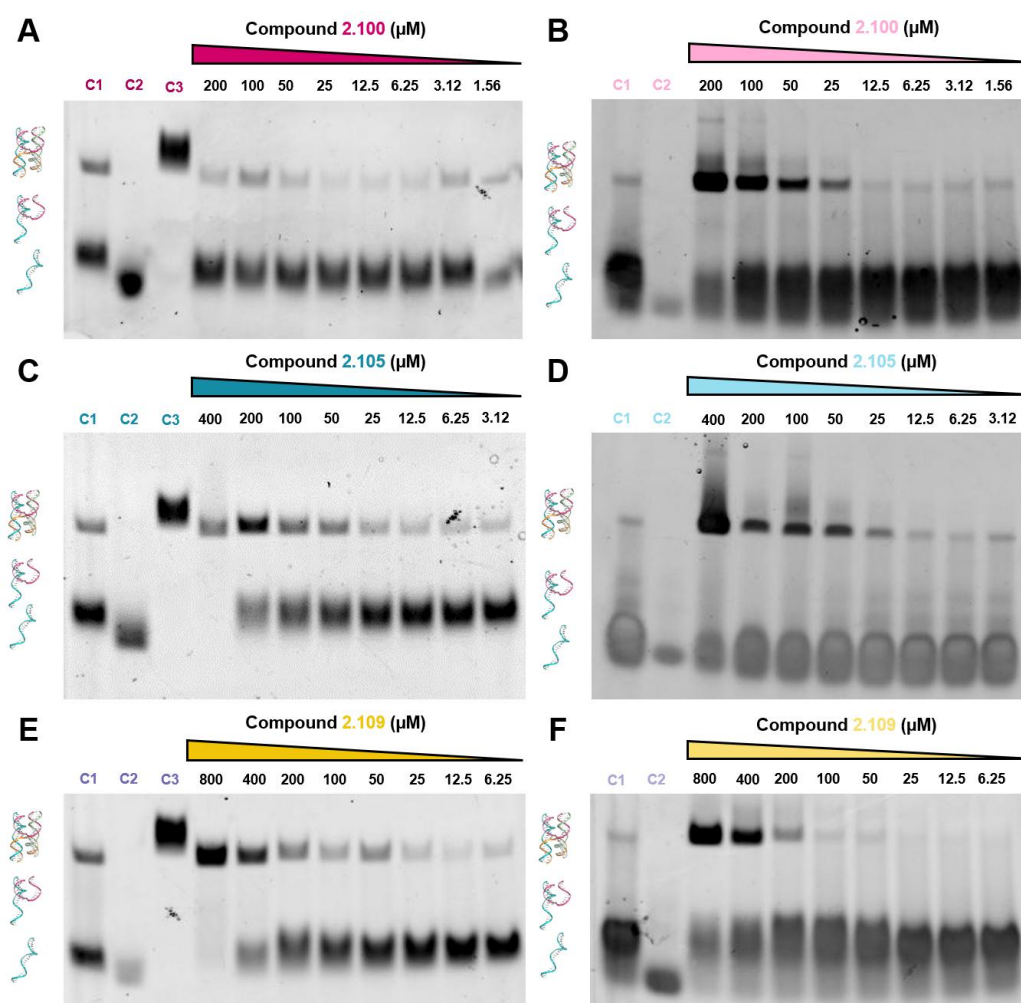


Figure 2.31: Gel images of compounds **2.100**, **2.105** and **2.109** in **A/C/E**. 4WJ trapping experiments, compound annealed with X, B, H, R strands overnight, 1:2 dilutions. **B/D/F**. 4WJ promotion experiments, compound incubated with X, B, H, R oligonucleotides for 1 hour at room temperature, 1:2 dilutions. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand, **C3:** 50 μM known 4WJ binding compound **2.18**. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

Gel electrophoresis was chosen to assess whether the rigidly designed acridine dimers **2.100**, **2.105** and **2.109** could bind to 4WJs. Three controls were included in each gel. Consistent with the previous experiments, the first two controls were kept (**C1**, **C2**), the third control (**C3**) is 50 μM of compound **2.18**, to confirm the location the 4WJ band appears. All three dimers showed promising 4WJ binding properties at high concentrations, which were explored as a minimal effect was seen with concentrations up to 200 μM . In the stabilising gels (**Figure 2.31A, C, E**), the required concentrations for binding is higher than the more flexibly linked dimers, likely due to the restrictions in bending to fit the most stable structure. Interestingly, the bands observed in the lower concentrations suggest that the dimers are inhibiting the 4WJ from forming, as the bands appear more faded than the control well.

Gel images of the 4WJ promoting assays (**Figure 2.31B, D, F**) produce an intense 4WJ band at concentrations down to 25 μM , significantly lower than in the trapping gels. Allowing the oligonucleotides to assemble in agreement with the compounds seems to lead to improved binding and formation of more stable 4WJ complexes.

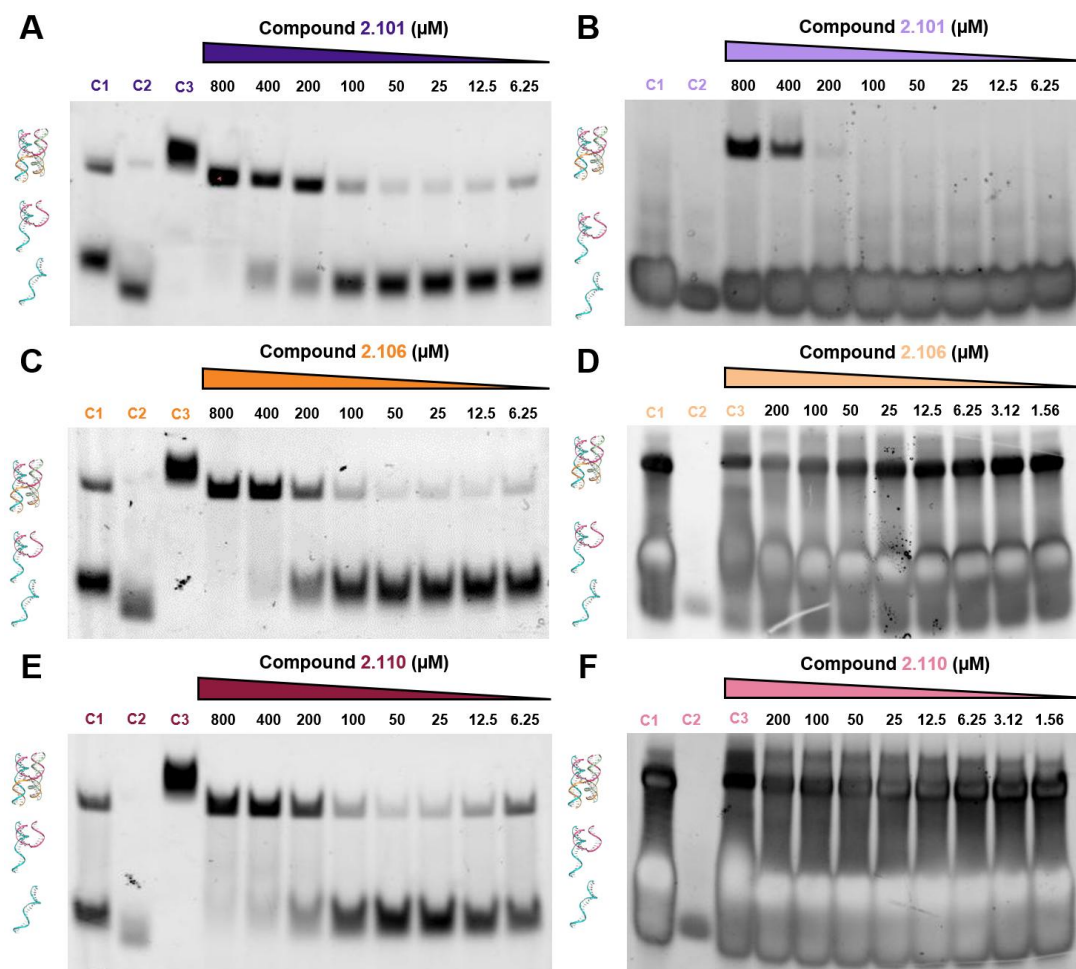


Figure 2.32: Gel images of compounds **2.101**, **2.106** and **2.110** in **A/C/E**. 4WJ trapping experiments, compound annealed with X, B, H, R strands overnight, 1:2 dilutions. **B/D/F**. 4WJ promotion experiments, compound incubated with X, B, H, R oligonucleotides for 1 hour at room temperature, 1:2 dilutions. **Controls C1:** Four oligonucleotides annealed at 1.25 μM in the absence of ligand, **C2:** 1.25 μM X strand, **C3:** 50 μM known 4WJ binding compound **2.18**. Both gels ran for 2 hours at 50 V, followed by staining with Sybr Gold stain for 30 minutes prior to imaging. All solutions were prepared in TBE buffer.

Compounds **2.101**, **2.106** and **2.110** were able to bind and stabilise 4WJs at high concentrations ($\geq 200 \mu\text{M}$), forming a clear junction band (**Figure 2.32A, C, E**). The promotion gels in **Figure 2.32D, F** produced an intense 4WJ band in the C1 controls, potentially due to preparation errors or overexposure, as a result interpretation of the compounds' effects is limited and experiment repetition is required.

The only two small molecules reported in literature with the ability to induce the formation of a 4WJ at room temperature are VE-822 and RTA, introduced in **sections 2.1.8** and **2.1.4**, accordingly.^{339,344} Evaluated through EMSA assays performed under the same conditions presented in this section, VE-822 promoted 4WJ formation down to 10 μM of compound, whilst RTA down to 30 μM . Additionally, an EMSA assay with palladium (II)/platinum cage complex discussed in **section 2.1.10**, reported

promotion of the 4WJ from 0.025 μM .³⁴⁸ Here, twelve small molecules are reported able to induce 4WJ formation at varying concentrations, with the lowest concentrations reached by **2.18** and **2.77** at 3.12 μM , and **2.75** at 1.56 μM .

In regard to 4WJ binding upon DNA annealing, the best junction binding compounds reported in the literature are peptides WRWYCR and WRWYRGGRYWRW, introduced in **section 2.1.2**. An EMSA assay performed of the two peptides reported 4WJ binding and stabilisation by WRWYRGGRYWRW as low as 10 nM and for the WRWYCR dimer 25 nM.³³³ From the tested acridine dimers, compound **2.18** performed the best in this assay carrying out 4WJ binding down to 1.56 μM , followed by compounds **2.75** and **2.77** at 3.12 μM .

2.3.14 Evaluation of four-way junction conformation upon ligand binding with a FRET assay

A crystal structure acquired by the Cardin and Searcey group, discussed in **section 2.1**, presented compound **2.18** bound to a 4WJ structure in the X-stacked conformation.³¹⁹ However, due to the complexity of acquiring a crystal structure of multiple compounds, a screening assay was developed using a Förster resonance energy transfer (FRET) system to investigate the preferred 4WJ conformation of the compounds upon binding. FRET is an energy transfer mechanism, carried out between two chromophores, one acting as a donor and the other as an acceptor. This system relies on the fluorescent properties of the chromophores, absorbing light at one wavelength, and emitting light at a different wavelength, named excitation and emission wavelengths, accordingly. In the absence of a FRET acceptor, when a molecule absorbs a photon, leading to an electron jumping to a higher state, once the electron returns to the ground state, it releases its energy as photon. However, in the presence of a FRET partner, the energy is transferred to another molecule, this action is called FRET.

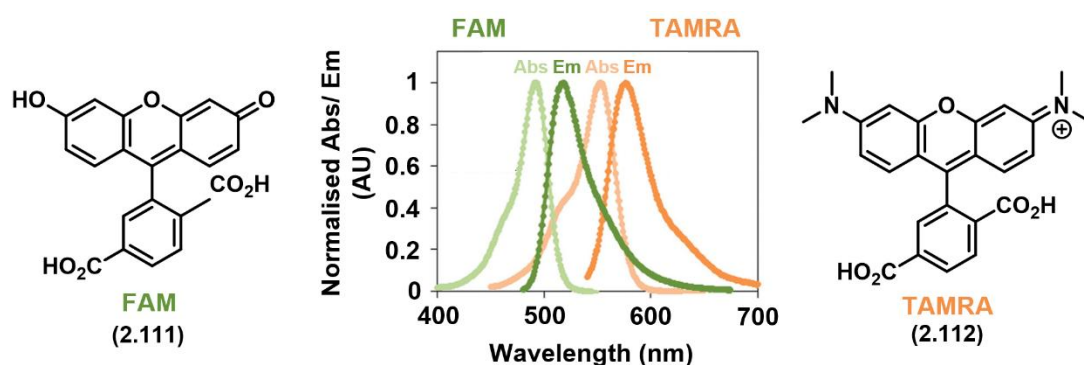


Figure 2.33: Spectral overlap of FRET partners, FAM (**2.111**) and TAMRA (**2.112**), used in the FRET assay. Plot published by Buckhout-White et al.³⁸⁶ Chemical structures of fluorophores.

In this assay, the chosen FRET partners are FAM and TAMRA. For FRET to occur, considerable overlap between the emission wavelengths of FAM and the excitation

wavelengths of TAMRA is required, allowing for the energy transfer. Figure **2.32** shows the absorption and emission of both fluorophores, highlighting the overlap.

A FRET signal is directly related to the distance between the fluorophores, as the Förster theory indicates that FRET efficiency (**E**) varies as the inverse sixth power of the distance between the two fluorophores (**r**).³⁸⁷ The relationship between FRET efficiency and distance **r** is presented in the following equation:

Equation 2.2: Förster equation highlighting relationship between FRET efficiency and fluorophore distance

$$E_{FRET} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

R_0 is defined as the Förster distance at which the efficiency of the FRET transfer is at 50%, it is expressed in angstroms and is defined as:

Equation 2.3: Förster distance equation

$$R_0^6 = (8.79 \cdot 10^{23}) \kappa^2 n^{-4} \Phi_\delta J_{da}$$

where κ^2 is the orientation factor, n represents the refractive index of the solvent, Φ_δ is the quantum efficiency and J_{da} is the overlap region of the donor emission spectrum with the absorption spectrum of the FRET acceptor.³⁸⁷ **Equation 2.2** highlights the direct relationships between FRET efficiency and the distance between the two fluorophores. The Förster distance for FAM and TAMRA is approximately 50 Å.³⁸⁸

The assay has previously been used to monitor conformational changes of a 4WJ upon the addition of different cations.³⁸⁹ One fluorophore is attached to the 5' terminus of an oligonucleotide, and the second one on the 5' terminus of a second one. As a result, when the 4WJ is in the X-stacked conformation, the two FRET partners are in close proximity, leading to an energy transfer. When in the open conformation there is no energy transfer, therefore no FRET signal is recorded. This technique is a reliable way to monitor conformational changes of a 4WJ upon binding of different compounds. The chosen 4WJ sequence differed to the one used in the gel electrophoresis experiment. A well-studied junction, J3, was chosen, as it has been shown to prefer isoform II, 77.4%, over isoform I, when in the X-stacked conformation, seen in **Figure 2.34**. FRET only occurs with isoform II, therefore, it is important for the majority of the 4WJ to undertake that isoform. Additionally, as a more stable 4WJ, it eliminates the interference of ssDNA and pseudo-duplexes.

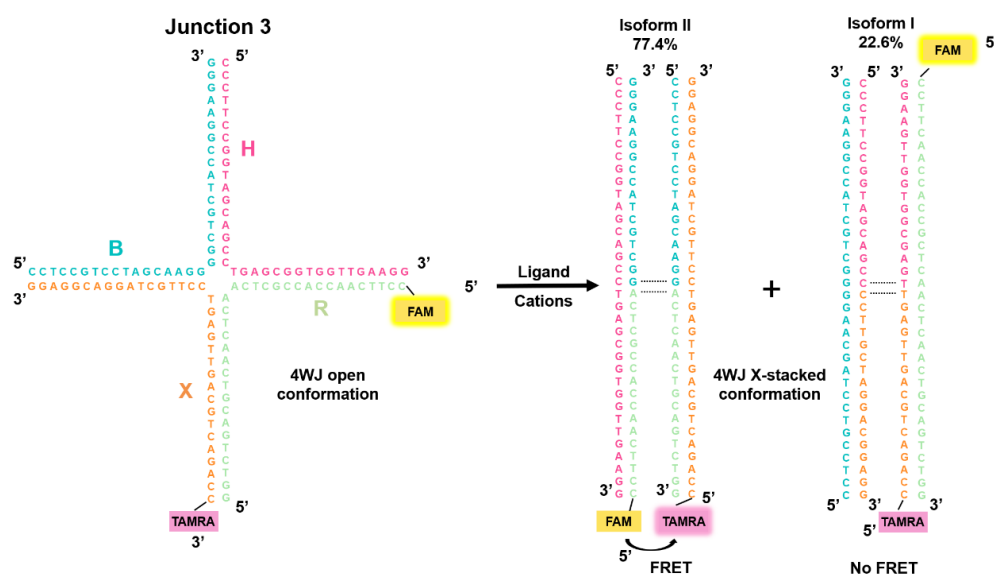


Figure 2.34: Junction 3 used in FRET experiment in open conformation, undertaking two isoforms of the X-stacked conformation upon addition of a ligand or cations

Oligonucleotides X, B, H and R were annealed overnight, forming the open conformation 4WJ structure. The absorption wavelengths of the two fluorophores and the acridines were taken into consideration for the selection of the excitation wavelength. The point of excitation must only excite FAM, avoiding both acridine and TAMRA excitation wavelengths. The chosen wavelength used in the experiments was 485 nm, reading the emission at 550 nm (FAM) and 600 nm (TAMRA). However, **Figure 2.33** highlights that TAMRA can be excited at 485 nm to a small degree, therefore a small percentage of the measured TAMRA emission measured during the experiment would be attributed to this. Additionally, there is a slight overlap between the TAMRA and FAM emission. As the main purpose of this assay is to investigate whether the acridine dimers will cause a conformation change of the 4WJ without a calculating the distance of the two fluorescent tags, a ratio of the two fluorophore emissions was measured and plotted against compound concentration, defined as:

Equation 2.4: FRET ratio of TAMRA emission over FAM emission

$$\text{FRET ratio} = \text{TAMRA}_{\text{Em}} / \text{FAM}_{\text{Em}}$$

As the 4WJ changes to the X-stacked conformation, the FAM emission decreases whilst the TAMRA emission increases, leading to overall increase in the FRET ratio. A ratio of the two fluorophore emissions is often used in Homogeneous Time-Resolved Fluorescence (HTRF) assays to indicate a change in the proximity of the two fluorophores upon addition of a ligand.^{390,391}

Titration of MgCl_2 was performed as a control experiment, showing an increasing FRET signal (**Figure 2.35A**), confirming the conformational change of the 4WJ.

Plotting the relative fluorescence against the concentration of ligand/cations required to reach a plateau, can be used to calculate a dissociation constant (K_D), corresponding to the concentration required to reach a 50% response. A K_D value of 28.5 mM was measured for the $MgCl_2$. The assay was performed in a 96 well plate, with each concentration performed in triplicate. All dimers were assessed, all twelve compounds inducing a conformational change. A proportional increase was measured between the FRET ratio and the compound concentration increase, confirming none of them bind to the open conformation of the 4WJ. Interestingly, the relative fluorescence upon addition of an acridine dimer reached considerably higher levels than the $MgCl_2$, suggesting a similar conformation is undertaken, however the fluorophores are placed in closer proximity, encouraging a stronger FRET signal.

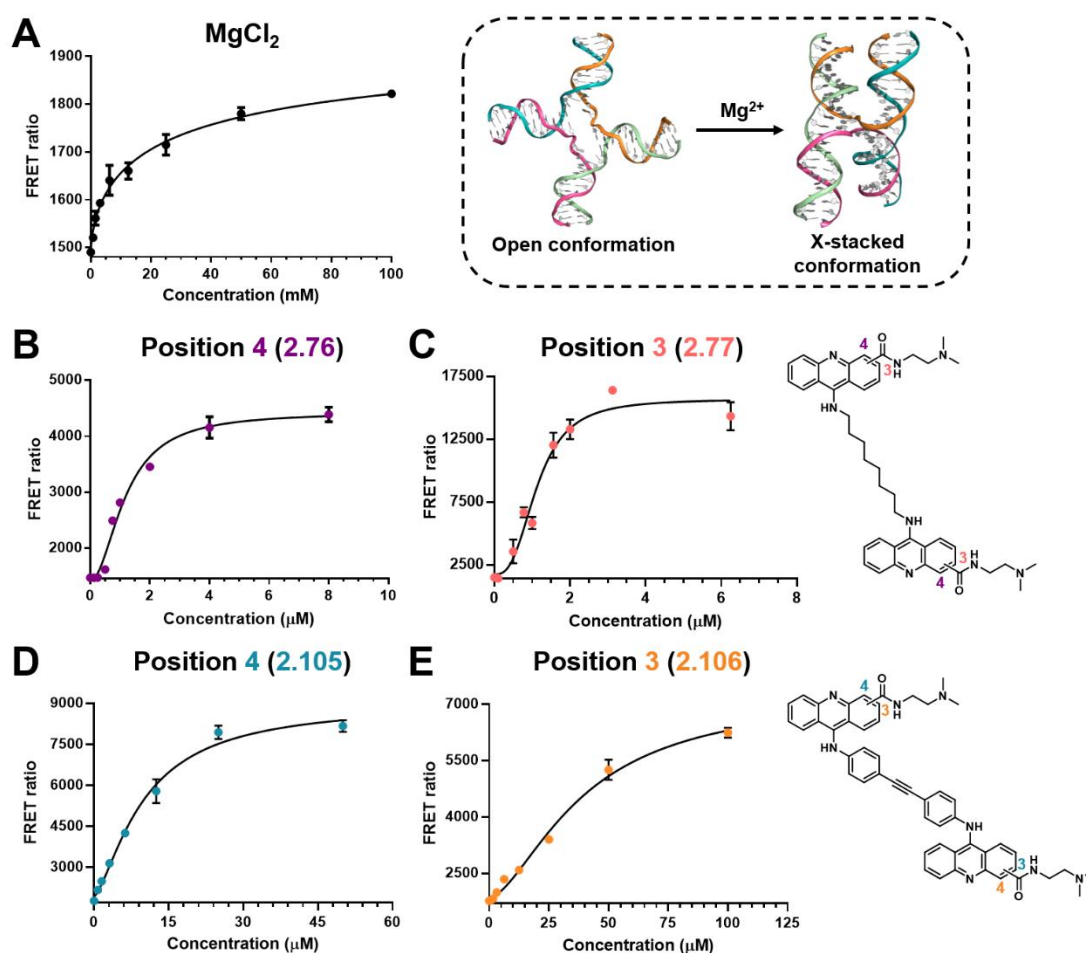


Figure 2.35: FRET ratio against concentration plots of doubly-labelled J3 with **A.** $MgCl_2$, **B.** **2.76**, **C.** **2.77**, **D.** **2.105** and **E.** **2.106**. Solutions were prepared in 10 mM Tris-HCl, 62.5 mM NaCl, pH 7.5, plots created with GraphPad using a dose-response binding model ($n=3$) ($\pm$ SEM).

K_D values were calculated for all compounds, highlighting compound **2.76**, requiring a concentration of $0.62 \pm 0.05 \mu M$ to reach 50% of the conformational change. Compounds **2.75**, **2.77** and **2.18** follow, with K_D values of 0.81 ± 0.08 , 0.90 ± 0.12 and $1.01 \pm 0.31 \mu M$ accordingly. The results complement the gel imaging, in which these

compounds required the lowest concentrations to trap and bind 4WJs. However, due to the difference in the sequences, these two experiments are not directly comparable.

Compounds **2.87** and **2.94** lead to a conformational change of the 4WJ with K_D values of 4.41 ± 0.42 and 1.59 ± 0.30 μM values accordingly. The three rigidly linked dimers bearing the carboxamide chain on position 4, require significantly lower concentrations to reach a conformational change when compared to their regioisomers (>40 μM). Detailed K_D values are presented in **Table 2.2**. Looking at **Figure 2.35D**, compound **2.105** has a K_D value of 10.06 ± 1.13 μM . Interestingly this signifies a compound to DNA ratio of 200 to 1, matching the gel electrophoresis results. This would support the hypothesis that a higher concentration of compound is required to bind to annealed 4WJ, demanding a conformational change to allow binding. However, when assembly of the 4WJ is solely induced by the compound, less rearrangement is required, folding the 4WJ in the most favourable conformation for binding.

Table 2.2: Dissociation constants (K_D) calculated for the acridine dimers through the FRET assay, observing the change in conformation of 4WJ J3, upon binding of the compounds ($\pm\text{SEM}$) ($n=3$).

Regioisomers	Compound	K_D (μM)	Compound	K_D (μM)
	2.18	1.01 ± 0.31	2.100	12.14 ± 2.12
	2.75	0.81 ± 0.08	2.101	48.59 ± 5.05
	2.76 	0.62 ± 0.05	2.105 	10.06 ± 1.13
	2.77 	0.90 ± 0.12	2.106 	39.40 ± 4.28
	2.87	4.41 ± 0.42	2.109	4.09 ± 0.65
	2.94	1.59 ± 0.30	2.110	55.75 ± 6.01

2.3.15 Investigation of four-way junction stability upon ligand binding with FRET-based melting assays

A FRET-based melting assay was carried out, evaluating the dimers' stabilising properties for duplex DNA, 3WJ and 4WJ DNA. This assay was first developed for the screening of chemical libraries, to investigate the interactions, between ligands and quadruplex structures.^{392–394} This was accomplished by measuring the change in the melting temperature of a quadruplex, induced by the presence of the ligand. Mergny and others^{392–398} have thoroughly exploited the use of this assay with quadruplexes, which was extended into 3WJ and 4WJs by Monchaud *et al.*^{399–405}

Junction forming oligonucleotides are doubly labelled with FRET partners, most commonly FAM and TAMRA, at both ends. The oligonucleotides chosen are designed

to allow close proximity of the two fluorophores when the DNA is in a folded state, leading to a FRET signal. The solution is subsequently heated, leading to DNA denaturation and the unfolding of the secondary structure. As a result, the monitoring of the FRET signal is a direct indication of the spatial proximity of the two partners, linked to the structural state of the complex (**Figure 2.36**).

Often in FRET-based systems, the emission read is that of the acceptor, in this case that would be TAMRA. However, when developing this assay, it was decided that monitoring of the fluorescent donor's emission, FAM, was more reliable in terms of reproducibility.³⁹² It is possible that other mechanisms other than FRET can lead to FAM quenching, therefore by reading the TAMRA emission there is no direct proportion between the signal and the denaturing level of the structure. Secondly, the overlap of FAM and TAMRA emission wavelengths would complicate isolating the changes in the FRET signal from the simultaneous FAM emission changes. Finally, it was reported that the majority of real-time PCR machines are unable to measure TAMRA emission in the required wavelengths when exciting at 490 nm.³⁹²

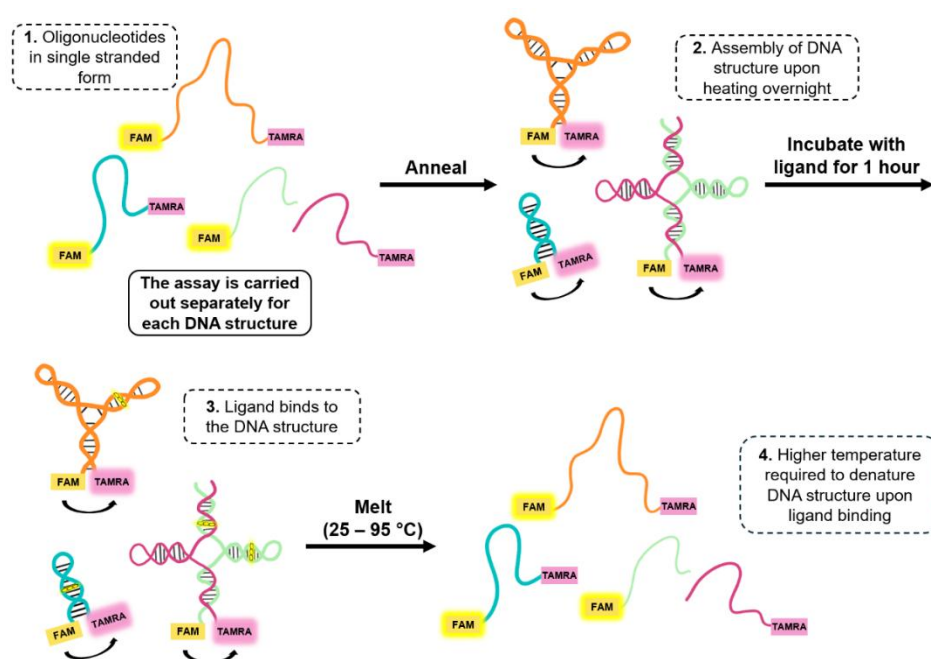


Figure 2.36: Graphical representation of the FRET-based melting assay. Starting with the oligonucleotides as ssDNA, assembling the secondary DNA structures upon annealing and allowing the ligand to bind, prior to heating the sample between 25 - 95 °C, increasing 1 °C per minute, until complete unfolding of the structures

As the absorbance of the fluorophores is measured in this assay, it was necessary to consider the absorbance of the compounds to ensure there was no wavelength overlap. The UV traces carried out for the FID assay, discussed in **section 2.3.9**, were consulted, showing minimal to no absorption at 490 nm from the dimers, assuming no interaction occurs between the fluorophores and the compounds during the assay.

It is important that the DNA structures chosen for this experiment are stable in their secondary forms, to lessen interference from unbound ssDNA molecules. To ensure this, the oligonucleotide sequences that were selected have been reported in literature for this assay.⁴⁰⁵ The use of the gel electrophoresis oligonucleotides was not possible, as a large amount of DNA does not form the 4WJ upon annealing, seen in the control well of the 4WJ trapping gels (for example in **Figure 2.26A**), which would affect the readings. Therefore, the selected duplex DNA and 3WJ structure were assembled from a single oligonucleotide each, strategically designed to be self-complementary in the required sections to make the appropriate shapes.⁴⁰⁵ This decision was made to eliminate any potential problems with multi-stage denaturation, due to individual strands breaking off the structure at different stages of the melting. The 4WJ was made up of two oligonucleotides, a compromise to limit conformational restrictions of the junction, allowing adaptations for preferred ligand binding.

The three DNA architectures were tested in the absence of any ligand to measure thermal denaturation, measured at 57 ± 1 , 50 ± 2 and 40 ± 2 °C, for the duplex, 3WJ and 4WJ DNA, respectively. Each ligand was prepared to a final concentration of 10 μ M, with the DNA at 200 nM, in 10 mM lithium cacodylate buffer, with 10 mM KCl and 90 mM LiCl at pH 7.2. This buffer was chosen as the pH does not change upon increasing the temperature, maintaining experiment conditions throughout the melting.⁴⁰⁶ This is important as secondary nucleic acid structures can be affected by pH variations.¹⁶⁷ The DNA was annealed overnight, to ensure the complementary sections had formed the appropriate structures. The DNA solutions were mixed with the ligand, transferring 20 μ L to PCR microtubes, and allowed to incubate for 1 hour. As the binding mode of the compounds to the DNA junctions is unclear, possibly binding either through intercalation or junction binding, by incubating the DNA with the ligands for 1 hour they are allowed to bind in the most stable mode prior to melting. Additionally, the incubation time mimics the gel electrophoresis assays, suggesting the same type of binding mode is being assessed in both assays. The experiment was carried out in a real-time PCR machine, therefore the samples were centrifuged whilst heating, ensuring even distribution of the ligand in the solution. The run began with 5 minutes at 25 °C, increasing by 1 °C every 1 minute, up to 95 °C, recording the FAM emission. Upon completion, the temperature of midtransition, T_m , was calculated for each DNA structure, in the presence of each of the acridine dimers.

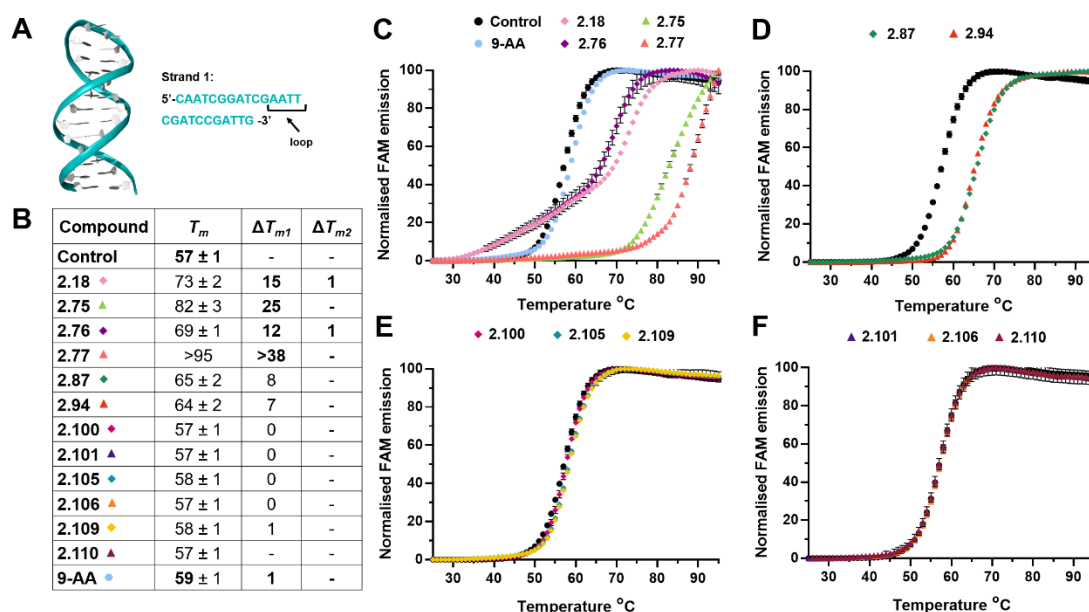


Figure 2.37: Melting experiment of dsDNA **A.** alphafold3 prediction of the self-complementary oligonucleotide, forming a duplex hairpin structure, **B.** table of melting temperature recorded of DNA structure in the presence of each acridine dimer, and control compound 9-aminoacridine. **C.** Graphs presenting the FAM emission recorded in the absence of ligand (control-black), in the presence of 9-AA (blue), and compound **2.18** (pink), **2.75** (green), **2.75** (purple) and **2.77** (red) **D.** **2.87** (green) and **2.94** (red), **E.** **2.100** (pink), **2.105** (blue) and **2.109** (yellow), and **F.** their regioisomers **2.101** (purple), **2.106**, (orange) and **2.110** (maroon). All experiments were run at 200 nM DNA, with the addition of 10 μ M ligand, in 10 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl at pH 7.2 ($n=3$) ($\pm$ SEM).

Additionally, 9-AA was also tested as a control compound. Small increases in stability were noted, with the duplex T_m only increasing by 1.78 $^{\circ}\text{C}$, consistent with literature⁴⁰⁷. The dsDNA molecule chosen for this assay is a self-complementary strand, that forms a hairpin structure when annealed (**Figure 2.37A**). This DNA structure was chosen to mimic the duplex section of the junctions chosen, to determine whether the presence of the rest of the junction alters the binding properties of the compounds. If the dimers bind to the DNA junctions solely through monointercalation, the same stabilisation would be expected to be noted for the hairpin structure.

Analysing the data recorded for duplex DNA stabilisation, presented in **Figure 2.37**, the data mostly agrees with the FID assay results. Flexibly linked compounds **2.18** and **2.76**, and their regioisomers **2.75** and **2.77**, significantly increase the denaturing temperature of the duplex at 10 μ M, ranging from 12 to 32 $^{\circ}\text{C}$. Interestingly, the two 9-aminoacridine-3-carboxamide dimers **2.75** and **2.77** performed best, with a ΔT_m of 25 and 32 $^{\circ}\text{C}$, suggesting the formation of a very stable complex upon binding to the duplex hairpin. The rigidly linked acridine dimers show little to no stabilisation of the duplex DNA, independent to the carboxamide side chain position. This complements the low binding affinity for ctDNA measured in the FID assay, indicating poor dsDNA binding affinity. Selectivity for secondary DNA structures over duplex DNA is one of the biggest issues with DNA intercalators, therefore these results show promise.

Compounds **2.87** and **2.94** show moderate stabilisation of approximately 8 °C, not affected by the position of the carboxamide chain.

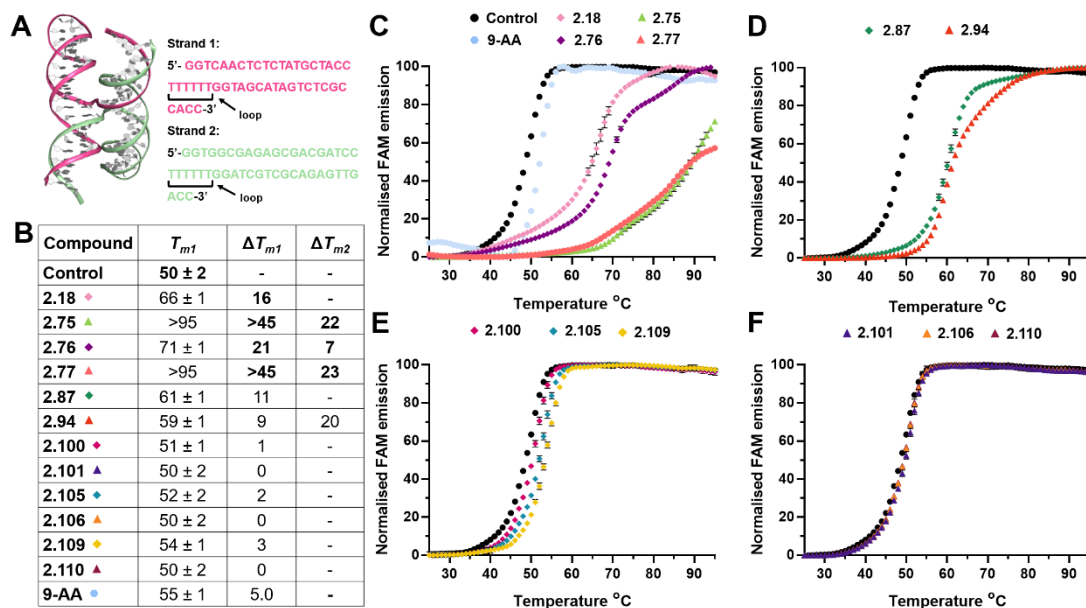


Figure 2.38: Melting experiment of 4WJ **A.** alphaFold3 prediction of the 4WJ structure assembled by the two labelled oligonucleotides, **B.** table of melting temperatures (T_m) recorded of DNA structure in the presence of each acridine dimer, and control compound 9-aminoacridine, and the difference between the T_m measured in the presence and the absence of ligand (ΔT_m). **C.** Graphs presenting the FAM emission recorded in the absence of ligand (control-black), in the presence of 9-AA (blue), and compound **2.18** (pink), **2.75** (green), **2.76** (purple) and **2.77** (red) **D.** **2.87** (green) and **2.94** (red), **E.** **2.100** (pink), **2.105** (blue) and **2.109** (yellow), and **F.** their regioisomers **2.101** (purple), **2.106** (orange) and **2.110** (maroon). All experiments were run at 200 nM DNA, with the addition of 10 μ M ligand, in 10 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl at pH 7.2 ($n=3$) ($\pm$ SEM).

The experiment was carried out with an annealed 4WJ structure (**Figure 2.38A**), under the same conditions. 9-AA mildly stabilised the junction by 5 °C, presumably due to intercalation into the duplex junction arms. Considerable increases were noted in the presence of several compounds. Compound **2.18** stabilised the 4WJ structure by 16 °C, similar to the reported ΔT_m for the duplex DNA. In contrast, compound **2.76** displayed notably higher stabilisation of the 4WJ, increasing the denaturing temperature by 21 °C. As seen in **Figure 2.38C**, the two regioisomers **2.75** and **2.77** stabilise the junction to a degree that prevents an accurate T_m calculation, with the increase in the melting temperature exceeding 45 °C. These results further support the strong binding interactions between these compounds and DNA, forming very stable structures. Additionally, compounds **2.76**, **2.75** and **2.77** lead to a double transition in the melting curve, suggesting two events are taking place, potentially due to stabilisation of an intermediate by the compounds once unfolding has begun. Good stability was observed for compounds **2.87** and **2.94**, with an average increase of 10 °C, with compound **2.94** also leading to two transitions during the melting ($\Delta T_{m2} = 20$ °C). When carrying the rigidly linked dimers through the assay, little stabilisation was

observed by the 4-carboxamide regioisomers **2.100**, **2.105** and **2.109**, with minor T_m increases up to 4°C. The change in the 4WJ sequence for this assay likely accounts for this observation, potentially suggesting some sequence selectivity from these compounds. Additionally, the incorporation of loops into the structure naturally changes the shape of the junction, potentially in an unfavourable conformation for the dimers to form stable complexes.

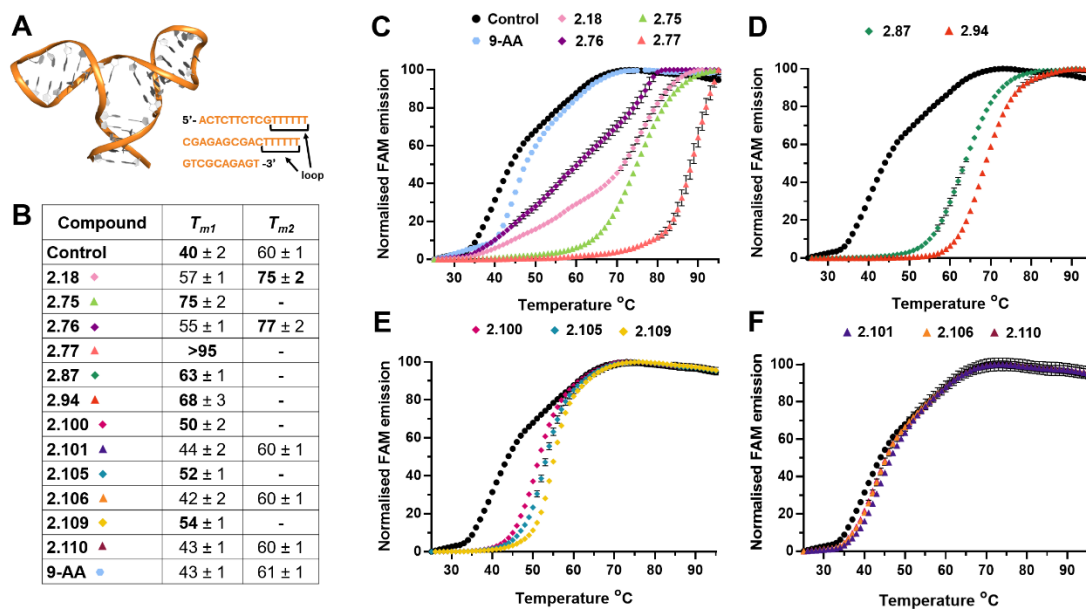


Figure 2.39: Melting experiment of 3WJ **A**, alphafold3 prediction of the self-complementary oligonucleotide, forming a 3WJ structure, **B**, table of melting temperature recorded of DNA structure in the presence of each acridine dimer, and control compound 9-aminoacridine. **C**, Graphs presenting the FAM emission recorded in the absence of ligand (control-black), in the presence of 9-AA (blue), and compound **2.18** (pink), **2.75** (green), **2.76** (purple) and **2.77** (red) **D**, **2.87** (green) and **2.94** (red), **E**, **2.100** (pink), **2.105** (blue) and **2.109** (yellow), and **F**, their regioisomers **2.101** (purple), **2.106** (orange) and **2.110** (maroon). All experiments were run at 200 nM DNA, with the addition of 10 μ M ligand, in 10 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl at pH 7.2 ($n=3$) ($\pm$ SEM).

The last DNA shape this assay was performed with was a three-way junction (**Figure 2.39A**). 3WJs differ from 4WJs in various structural elements, including the trigonal versus tetragonal central pocket, as well as the distance between the junction arms. It was of interest to investigate how the acridine dimers performed when evaluated against this DNA structure. The melting curve of the 3WJ in the absence of a ligand indicates there are two events occurring during the unfolding of the structure (T_{m1} = 40 °C, T_{m2} = 60 °C), interestingly, some of the compounds are able to shift this equilibrium into one transition upon binding. The temperature required to unfold the 3WJ structure was increased in the presence of all dimers, to varying degrees, as seen in the summary table in **Figure 2.39B**. The addition of 9-AA led to small increase of 3 °C, similar to the recorded values for dsDNA and 4WJ. A remarkably high increase in the T_m values was recorded for compounds **2.18**, **2.75**, **2.76** and **2.77**, doubling the melting temperature of the junction. The change between the positions

3 and 4 for the carboxamide does not seem to reduce the stabilising effects, with all four dimers performing very well. Compounds **2.18** and **2.76** are also characterised by two transition during the melting, however the shape of the curve suggests they may differ to the ones observed in the control experiment. Strong stabilisation was noted from compounds **2.87** and **2.94** with the 3-carboxamide regioisomer (**2.94**) outperforming the 4-carboxamide one (**2.87**). The rigidly linked 4-carboxamide dimers also performed well in this assay, as seen in **Figure 2.39E**, with ΔT_m values calculated between 10-15 °C, indicating considerably higher stabilisation of this 3WJ, compared to the dsDNA and the 4WJ. It is likely the distance between the junction arms is more suitable for the linear nature of these ligands, providing a better fit. The binding of **2.100**, **2.105** and **2.109** all shift the melting to a single transition event, potentially altering the conformation of the structure upon binding.

There are only two reports of melting experiments investigating 4WJ stability upon binding of a ligand in literature, assessing compounds TMPyP4 and the platinum cage introduced in **sections 1.4.2.3** and **2.1.10**, respectively. A FRET-based melting assay carried out under the same conditions as the experiments in this section reported that TMPyP4 increased the melting temperature of the 4WJ by 7 °C at 1 μ M.⁴⁰⁵ Furthermore, the Pt cage complex has a reported ΔT_m of 11 °C at 1 μ M, measured through a UV melting experiment.³⁴⁸ Here, solutions were treated with 10 μ M of ligand, limiting comparison of the results to literature. The highest stabilisation of a 4WJ was reached by compounds **2.75** and **2.77** with ΔT_m values higher than 45 °C, followed by compounds **2.18** and **2.75** with values of 16 and 20 °C accordingly. These are the highest melting temperatures recorded for a 4WJ structure bound to a ligand to date to our knowledge.

2.3.16 Investigation of four-way junction selectivity with competition melting assay

This assay assessed the stabilising abilities of these compounds under ideal conditions, directly measuring the effect they have on a DNA structure in isolation. However, considering the complexity of a cellular environment, the ligand must be able to selectively bind to the desired DNA structure, in the presence of other DNA shapes. To investigate this ability, a competition assay was developed, based on the same FRET principle, with the addition of an unlabelled secondary DNA structure to the solution. This will allow comparison of the two DNA architectures, without disturbing the readings of the FRET signal. A decrease of the labelled DNA's melting temperature, in the presence of the secondary structure, suggests low selectivity of

the compound. To ensure direct comparison, under the exact conditions, the assay was repeated both in the presence and the absence of the secondary DNA structure.

The 4WJ structure was tested in the presence and the absence of dsDNA and the 3WJ structure. A 1:1 ratio of labelled 4WJ and unlabelled dsDNA were used in solution, however no major effects were observed when compared to results gathered in the absence of dsDNA. Due to the excess of ligand used in these assays, 10 μ M, the equivalents of the DNA competitor were increased to 10, to observe whether the ligands could selectively bind to the 4WJ in the presence of excess dsDNA. This experiment was also carried out with a 3WJ competitor, to assess junction selectivity.

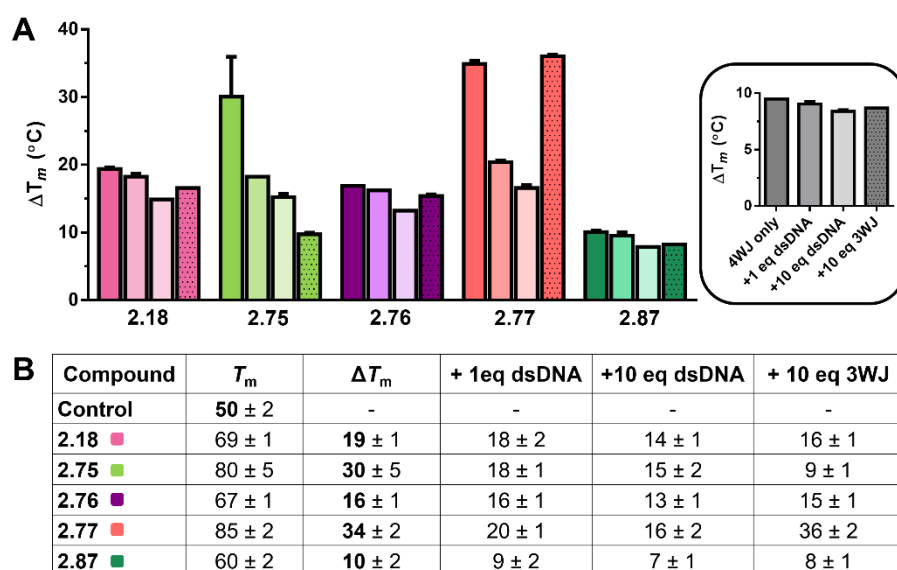


Figure 2.40: **A.** Results of competitive melting experiment, performed with 0.2 μ M 4WJ stability in the presence of 10 μ M compound **2.18**, **2.75**, **2.76**, **2.77**, and **2.87**, with the addition of 1 (medium gradient) or 10 (light gradient) equivalents of unlabelled dsDNA, or 10 equivalents of unlabelled 3WJ (dotted bar). **B.** Melting temperature results from the competition assay ($n=3$) ($\pm$ SEM).

The results are summarised in **Figure 2.40**, highlighting the change in the calculated ΔT_m of the 4WJs, with the addition of competing DNA structures. Dimers **2.18** and **2.76** show good selectivity for 4WJ, as only a 3 $^{\circ}$ C drop was measured in the presence of dsDNA, even less with the addition of 3WJ. In contrast, their regioisomers **2.75** and **2.77** show a considerable decrease in temperature upon addition of 1 equivalent of dsDNA, indicating their lack of selectivity for DNA structures. Lastly, compound **2.87** showed minimal changes in the melting temperature in the presence of dsDNA and 3WJ, maintaining good 4WJ stabilisation and suggesting good selectivity.

The assay was further used to investigate 3WJ selectivity, with unlabelled dsDNA and 4WJ as competitors. Similarly to the results shown in **Figure 2.41**, minimal changes were observed in the presence of 1 equivalence of dsDNA.

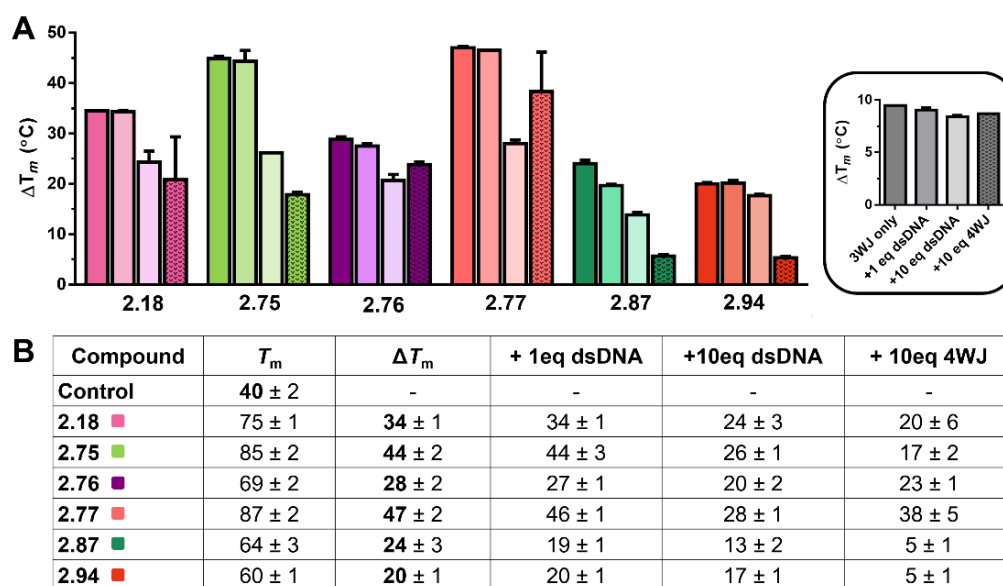


Figure 2.41: A. Competition melting experiment, showing the change in $0.2 \mu\text{M}$ 3WJ stability in the presence of $10 \mu\text{M}$ compound **2.18**, **2.75**, **2.76**, **2.77**, **2.87** and **2.94**, with the addition of 1 (medium gradient) or 10 (light gradient) equivalents of unlabelled dsDNA, or 10 equivalents of unlabelled 4WJ (dotted bar). B. Melting temperature results from the competition assay ($n=3$) ($\pm\text{SEM}$).

Upon addition of higher equivalents of DNA competitors, decreases in the melting temperature of the 3WJ were measured, for compounds **2.18**, **2.75** and **2**. Whilst the decrease corresponds to less selectivity for 3WJs, the ΔT_m values remain considerably high, over 18°C at $10 \mu\text{M}$, highlighting the promise of these compounds. DNA competitors have little effect on compound **2.75**, highlighting good stability. The 3WJ complex upon binding of regioisomers **2.87** and **2.94** is not majorly destabilised in the presence of dsDNA. However, a large decrease is observed with the addition of 4WJ, agreeing with the results discussed above that these compounds exhibit good 4WJ selectivity.

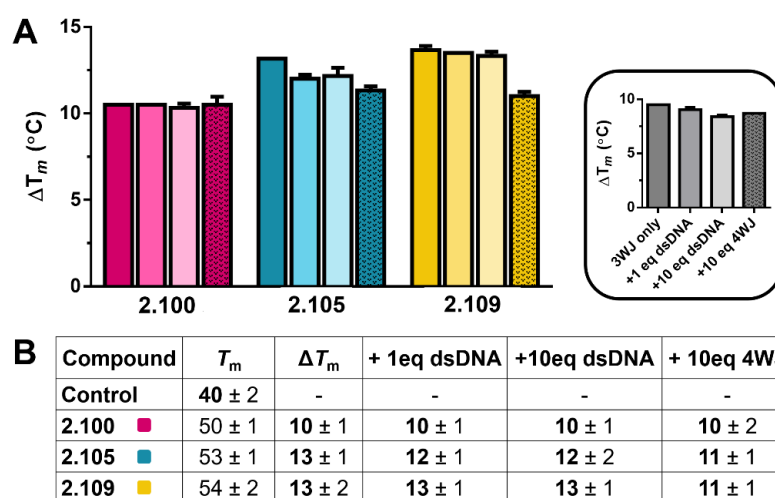


Figure 2.42: Competition melting experiment, showing the change in $0.2 \mu\text{M}$ 3WJ stability in the presence of $10 \mu\text{M}$ compound **2.100**, **2.105**, and **2.109**, with the addition of 1 (medium gradient) or 10 (light gradient) equivalents of unlabelled dsDNA, or 10 equivalents of unlabelled 4WJ (dotted bar). B. Melting temperature results from the competition assay ($n=3$) ($\pm\text{SEM}$).

The competition assay highlighted three very promising compounds, **2.100**, **2.105** and **2.109**. These compounds resulted in minimal to no stabilisation effects when assessed against dsDNA and 4WJ (**Figure 2.42**), however led to a 10 - 15 °C increase against the 3WJ. This apparent selectivity was further confirmed when directly competing the 3WJ with the other two structures, leading to no change in the results. Both assay results complement each other, further supporting the proposal that these compounds exhibit stable binding selectively with the 3WJ.

2.3.17 Measurement of DNA binding constants via fluorescence titration

A fluorescence quenching assay was performed, allowing direct comparison of the acridine dimers' binding affinities for duplex, 4WJ and 3WJ DNA. This assay is based on the characteristic of acridine fluorescence quenching occurring upon binding to DNA. It has been well studied that strong binding of acridine to nucleic acids leads to conformational changes in the molecule, resulting in a decrease in fluorescence.⁴⁰⁸ An investigative study into the cause of quenching of an acridine derivative, proflavine, uncovered that guanine was responsible for the fluorescence quenching.⁴⁰⁹ Spectrophotometric titration is one of the techniques used to study acridine derivatives as fluorescent molecules. Therefore, there is good evidence that the DNA binding affinity of an acridine derivative to a nucleic acid is directly proportional to the fluorescence quenching recorded upon binding. This allows for binding constants to be calculated for different DNA structures by carrying out titration experiments.

The DNA structures used in the FRET-melting experiment were chosen for the purpose of this assay, carrying no labels to prevent interference with the acridine fluorescence recordings. It is important for the experiment that the DNA junctions are stable in their assembled form, avoiding ssDNA or Y fork duplexes in the solution, obscuring the results.

A final concentration of 1 μ M was chosen for the acridine dimers, exciting the compounds at 400 nm, following previous excitation experiments, reading the emission between 415-600 nm. This region covers the emission spectra of the dimers, avoiding the DMSO signals located at lower regions. From the recorded spectra of the dimers in the absence of DNA, the signal peak was pinpointed between 440-450 nm, the wavelengths later used for binding constant calculations. To reach full quenching of the fluorescence the DNA titrations were gradual, with a total of 12 DNA titrations of 1 μ L. As the decrease in fluorescence intensity is rapid, the final DNA concentrations used started at 0.1 μ M increments, increasing to 0.25 μ M, 0.5 μ M and

finally 1 μM , to ensure all compound was bound to DNA. The titration graphs for compound **2.18** are presented below in **Figure 2.43**. To calculate the K_D values for the compound, the intensity values recorded at 444 nm during the titration were used to calculate the relative fluorescence. This was done using the equation:

Equation 2.5: Relative fluorescence equation used for fluorescence titration experiments

$$\text{Relative Fluorescence} = 1 - (F/F_0)$$

F stands for the fluorescence intensity recorded for each titration, F_0 is constant as it refers to the fluorescence intensity recorded at 444 nm in the absence of any DNA. Carrying out this equation for all the titrations with the three DNA structures allows for graph D. The binding curve was fitted to a one-site binding model using Graphpad Prism, allowing calculation of the binding constants.

The results demonstrated that compound **2.18** interacts with duplex DNA with a K_D of 4.23 μM , 2.07 μM with 3WJ and 0.28 μM with 4WJ, measured under the same conditions. Almost a 15-fold difference is recorded between the 4WJ and duplex DNA binding, and a 7-fold preference for 4WJ over 3WJ. Therefore, this compound shows strong binding to the 4WJ structure, complemented by the good stability recorded in the melting experiment, further supported by the small effect of the competing DNA molecules in the presence 4WJ.

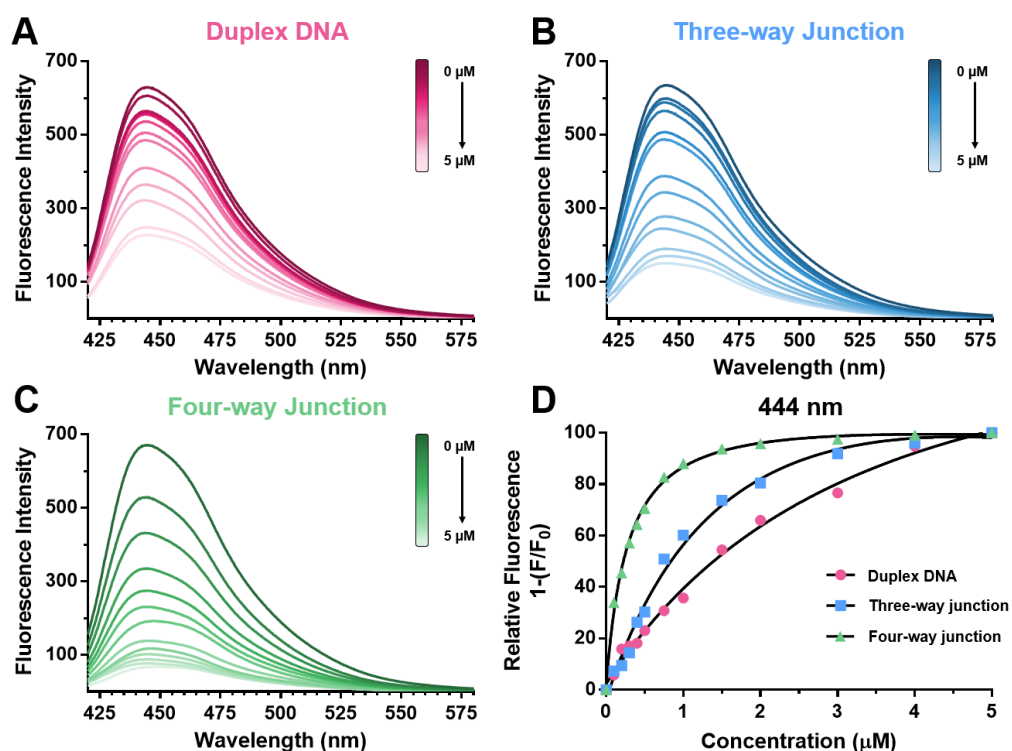


Figure 2.43: Spectral scans of 1 μM compound **2.18** upon addition of 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 μM of **A.** duplex DNA, **B.** 3WJ, **C.** 4WJ. **D.** Dose response curves plotted of the fluorescence intensity recorded for each experiment at 444 nm, plotted in GraphPad Prism fitted to a one-site model ($n=3$). Experiments were carried out in 20 mM Tris·HCl, pH 7.4.

The experiment was repeated for the remaining compounds, against all three DNA structures, calculating binding constants in the same way.

Table 2.3: Binding constants calculated from fluorescence titration results of acridine dimers with duplex DNA, 3WJ and 4WJ DNA, using GraphPad Prism, fitted to a one-site binding model, $Y = B_{max} * X / (K_d + X) + NS * X + Background$ ($n=3$) ($\pm SEM$).

Compound	Binding constants (nM)		
	dsDNA	3WJ	4WJ
2.18	344 ± 25	472 ± 54	90 ± 27
2.75	277 ± 34	840 ± 127	436 ± 28
2.76	620 ± 105	178 ± 12	167 ± 9
2.77	287 ± 46	680 ± 154	73 ± 13
2.87	337 ± 110	638 ± 123	258 ± 27
2.94	172 ± 34	627 ± 99	524 ± 40
2.100	113 ± 21	618 ± 94	211 ± 32
2.101	1,006 ± 239	247 ± 34	291 ± 33
2.105	277 ± 67	653 ± 166	143 ± 4
2.106	2,749 ± 391	1,363 ± 231	4,325 ± 126
2.109	263 ± 76	684 ± 175	141 ± 5
2.110	4,325 ± 954	11,580 ± 680	729 ± 116

Compound **2.77** exhibits the strongest binding to the 4WJ, with a 70 nM binding affinity, closely followed by compounds **2.76**, **2.100**, **2.105** and **2.109**, around 150 nM. Interestingly, the rigidly linked dimers showed good binding to the 4WJ. In relation to the 3WJ, good binding is measured, around 0.6 μ M. Additionally, compounds **2.101**, **2.106** and **2.110** exhibit the lowest binding to dsDNA.

The DNA sequences used in this assay were also used in the FRET-based melting assay, to investigate any correlation between the binding constant and stability measured. However, the results gathered are conflicting, showing low K_D values for compounds with no effect on stabilisation, such as compounds **2.100**, **2.105** and **2.109**. The varied lengths of the DNA structures must be taken into consideration, the 4WJ is considerably larger than the 3WJ and the dsDNA, potentially including more binding sites for the molecules. Additionally, this assay measures an instant response, it is likely the binding constants are representative of the acridines solely intercalating into the DNA, with junction binding requiring more time to account for conformation changes of the junction to the preferred binding. These results would be well complemented by stoichiometry data which can be determined through determining Job's plots, confirming the recorded data is associated with 1 to 1 binding of a

compound to DNA. Due to these limitations, the binding constants of a compound with the different DNA structures should not be compared, whereas the compounds may be compared to each other for each DNA structure separately.

The binding constants measured for the acridine dimers in relation to 4WJ show great promise, with the compounds binding to the DNA structure in the nanomolar range. Comparing to literature, all dimers except **2.106** outperform the Pt cage complex with a reported binding constant of 827 nM.³⁴⁸ The best 4WJ binding compound described here is **2.77** with a 70 nM binding constant.

2.4 Conclusions

This chapter presents the design and synthesis of twelve 9-aminoacridine-3/4-carboxamide dimers. The set of acridines synthesised covered four-flexibly-linked compounds (**2.18**, **2.75**, **2.76**, **2.77**), one of which, compound **2.77**, has not previously been studied. This was complemented by two semi-rigid, “click”-derived compounds (**2.87**, **2.94**) that have previously been described. Finally, six rigidly linked compounds (**2.100**, **2.101**, **2.105**, **2.106**, **2.109**, **2.110**), with different linker lengths and orientations, complete the set of compounds and are novel diacridines. The compounds were assessed in a series of biophysical assays, investigating their binding to DNA junctions, their effect on junction stability and selectivity over duplex DNA.

Dimers **2.18**, **2.75**, **2.76** and **2.77**, all demonstrated similar behaviours. Good duplex DNA binding was observed in the FID assay, further supported by their ability to crosslink duplex-to-duplex DNA in the nanoparticle assay. They bind to 4WJs and can induce their formation at low concentrations (3.12 μ M), observed in the gel electrophoresis experiments, with the 4-carboxamide structure selectively forming only one 4WJ structure. Nanomolar range concentrations were required to cause a conformation change in the J3 4WJ measured in the FRET assay, indicating they are unable to bind to the open conformation. The FRET-based melting experiments measured significant stabilising effects on 3WJ and 4WJ DNA, with ΔT_m values >45 °C. However, the competition melting assay indicated no binding selectivity to 4WJs over 3WJs and duplex DNA.

Two semi-rigid dimers were assessed, exhibiting mild duplex DNA binding in the FID assay, but able to crosslink in the AuNP assay. The PAGE experiments suggest good binding to four-way junctions, able to trap them upon annealing and induce their formation at room temperature, however at higher concentrations than the flexibly-

linked dimers (25 μ M). Good stabilisation was recorded for 3WJ and 4WJ DNA, with the competition assays suggesting some selectivity for 4WJs over 3WJ and duplex DNA.

The 3-carboxamide rigid dimers **2.101**, **2.106** and **2.110** demonstrated poor binding and no stabilisation of duplex DNA, whilst requiring higher concentrations than the rest of the compounds to crosslink two DNA molecules. Additionally, they exhibit poor stabilisation of all three DNA structures in the melting experiments. They can bind to 4WJ structures at high concentrations, exhibited by gel electrophoresis and cause a conformation change upon binding to the junction. The shift of the carboxamide chain largely impacted the DNA binding abilities of these compounds in combination with the rigid linkers.

A considerable difference was observed in assay results of the rigidly-linked dimers bearing the carboxamide chain on position 4 than position 3. The 4-carboxamide rigid dimers **2.100** and **2.105** promote the formation of the 4WJ at low concentrations (25 μ M), with compound **2.109** requiring higher concentrations (200 μ M). The three dimers can crosslink DNA, however, display low binding to duplex DNA in the FID assay and no stabilisation of duplex DNA in the melting assay, suggesting they are poor intercalators. The melting experiments demonstrated minimal stabilisation of the 4WJ and selective stabilisation of the 3WJ, unaffected by the addition of other DNA structures. When tested in 3WJ gels the dimers were unable to induce the formation of the junction, suggesting they are unable to promote the assembly of a 3WJ however can bind well to a preformed structure. Additionally, a considerable difference was observed in the 4WJ PAGE experiments, when the oligonucleotides were annealed with the dimers, in comparison to allowing them to incubate at room temperature. These results suggest that they dimers exhibit improved binding when allowed to direct the junction assembly at room temperature, instead of inducing it with heat. This hypothesis complements the melting and FRET results, showing minimal stabilisation of a pre-formed junction and requiring high concentrations to induce a conformation change of the junction.

Chapter 3
The design and synthesis of
acridine trimers to target three-way
DNA junctions

3.1 Introduction

3.1.1 Three-way junction binding compounds

Three-way junctions (3WJs), also known as slippage loops or S-DNA, can form from direct repeat or inverted repeat sequences (IRS), which are abundant in the human genome and have been associated with genetic disorders.^{271,272} As mentioned in **section 1.4.6.3.1**, four-way junctions can also form in the presence of IRS, however if the hairpins that form are misaligned, it results in the formation of two 3WJs (**Figure 3.1**). IRS are present in key regions of the genome, allowing junctions to form.⁴¹⁰ Stabilisation of the 3WJs could prevent enzymes involved in key DNA processes from physically overcoming the junctions. As a result, enzymes such as topoisomerases and polymerases would be indirectly inhibited by the junction-barrier, with such blockage disrupting DNA replication and transcription, respectively, causing DNA damage.⁴¹¹ This concept led to a new strategy of targeting three-way junctions with small molecules to cause DNA damage in cells.

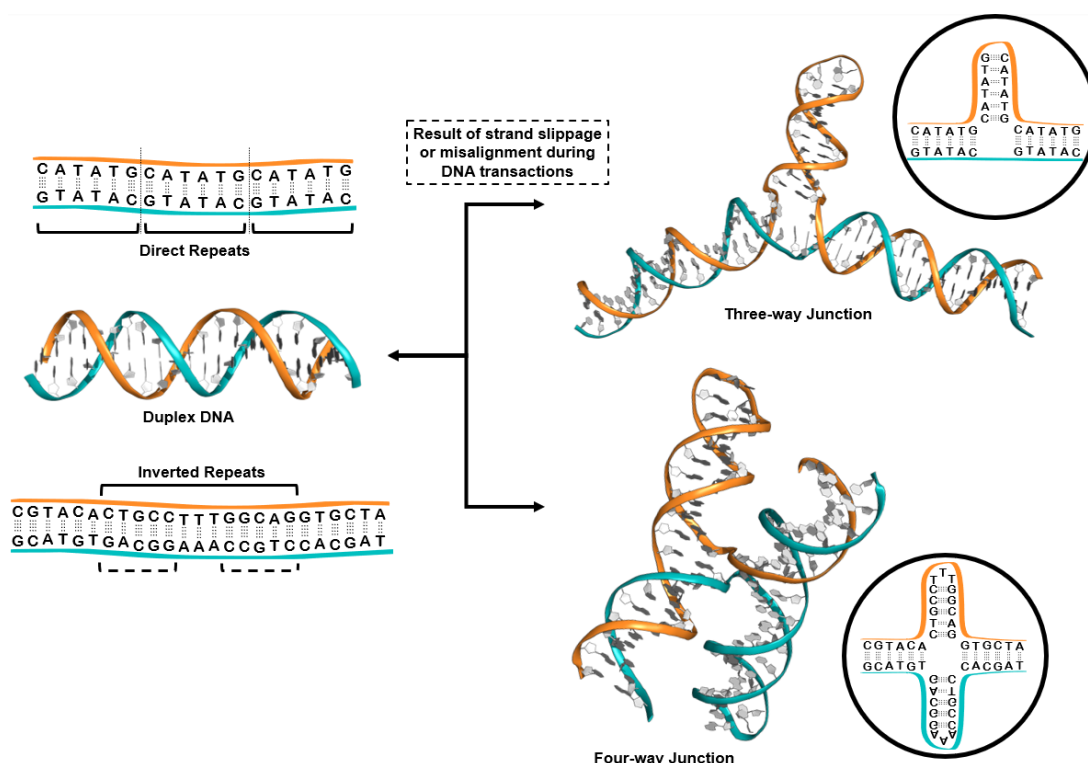


Figure 3.1: Representation of how duplex DNA containing direct repeats or inverted repeats can fold into three-way and four-way junctions accordingly

Initial work was carried out by Kallenbach *et al.* in an effort to target 3WJs with iron and copper complexes, highlighting the central cavity of the junction as a potential binding site for drugs.⁴¹² The first examples of small molecules targeting 3WJ were in the field of aptamers. Three-way junction aptamer sensors were designed to identify

drugs and steroids by binding them into the branch point of the structure.^{413–416} While in these examples the aptamers were designed to identify relevant compounds, the opposite strategy, to design small molecules that can bind to 3WJs, gained attention following the initial work by the Hannon group.⁴¹⁷

3.1.2 Supramolecular helicates

The first successful identification of a 3WJ binder was demonstrated by Michael Hannon, Miquel Coll and co-workers in 2006, with the publication of an X-ray crystal structure of a synthetic supramolecular helicate bound to the central cavity of a three-way junction (Figure 3.2).⁴¹⁷ The 3WJ-binding compound was made up of three bis-pyridyl ligands (3.1) wrapped around two Fe^{II} ions, forming a tetracationic supramolecular helicate [Fe₂L₃]⁴⁺. This helicate was initially investigated in relation to dsDNA, however, upon crystallisation with palindromic sequence 5'-CGTACG-3' it promoted the assembly of a Y-shaped 3WJ (Figure 3.2).

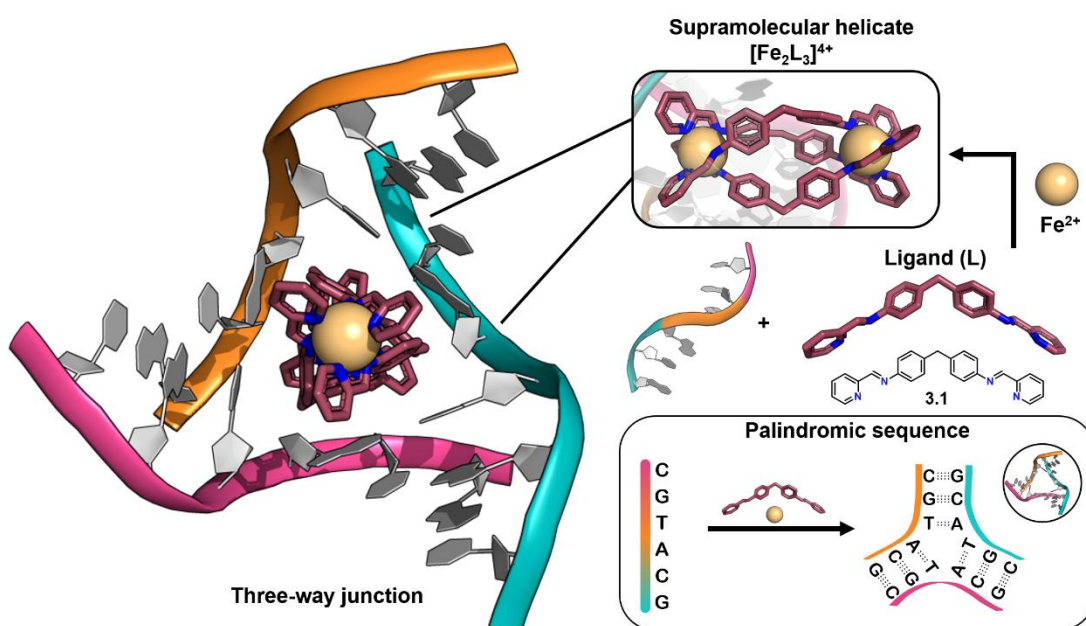


Figure 3.2: Crystal structure of a supramolecular helicate bound in the central cavity of a three-way junction made up of the palindromic sequence CGTACG

To confirm the helicate binding to the 3WJ was not an artifact of crystallisation, a set of gel electrophoresis assays were carried out, confirming the ability of the helicate to trigger the assembly of a 3WJ with non-palindromic oligonucleotides.⁴¹⁸ Further X-ray structure analyses and a thorough NMR study confirmed the potential of helicates as 3WJ-targeting compounds, initiating a new field of research.^{419,420} This new class of supramolecular structures is characterised by a general [M₂L₃]⁴⁺ motif, where ligands with metal-chelating features can assemble as trimers wrapped around two divalent cations.

Following from the successful characterisation of $[\text{Fe}_2\text{L}_3]^{4+}$ as a 3WJ binder, variations of this helicate have been explored to further improve its binding. Nickel and ruthenium versions of the cylinder were investigated as they are more stable than the iron analogue, and were found to maintain their 3WJ-binding properties.⁴²¹ A “switch on and off” system for cylinder $[\text{Ni}_2\text{L}_3]^{4+}$ was explored by wrapping a rotaxane around it, preventing it from binding to DNA (**Figure 3.3A**). This strategy was designed to create a system which is initiated by the removal of the rotaxane, switching on the mode of action of the cylinder.⁴²¹ The nickel cylinder was also assessed through stability and polyacrylamide gel electrophoresis (PAGE) assays in relation to different 3WJ central cavity structures, resulting in a clear Y-shaped symmetrical 3WJ binding preference. Additionally, a DNA polymerisation arrest assay highlighted the cylinder’s ability to halt DNA synthesis by binding to a 3WJ, showing the potential for 3WJs as therapeutic targets. Finally, a cell viability assay assessed the cytotoxicity of the cylinders, associating the mode of action with DNA damage, further supported by a combination assay with DNA repair inhibitors, showing synergism, with combination index values as low as 0.62.⁴²²

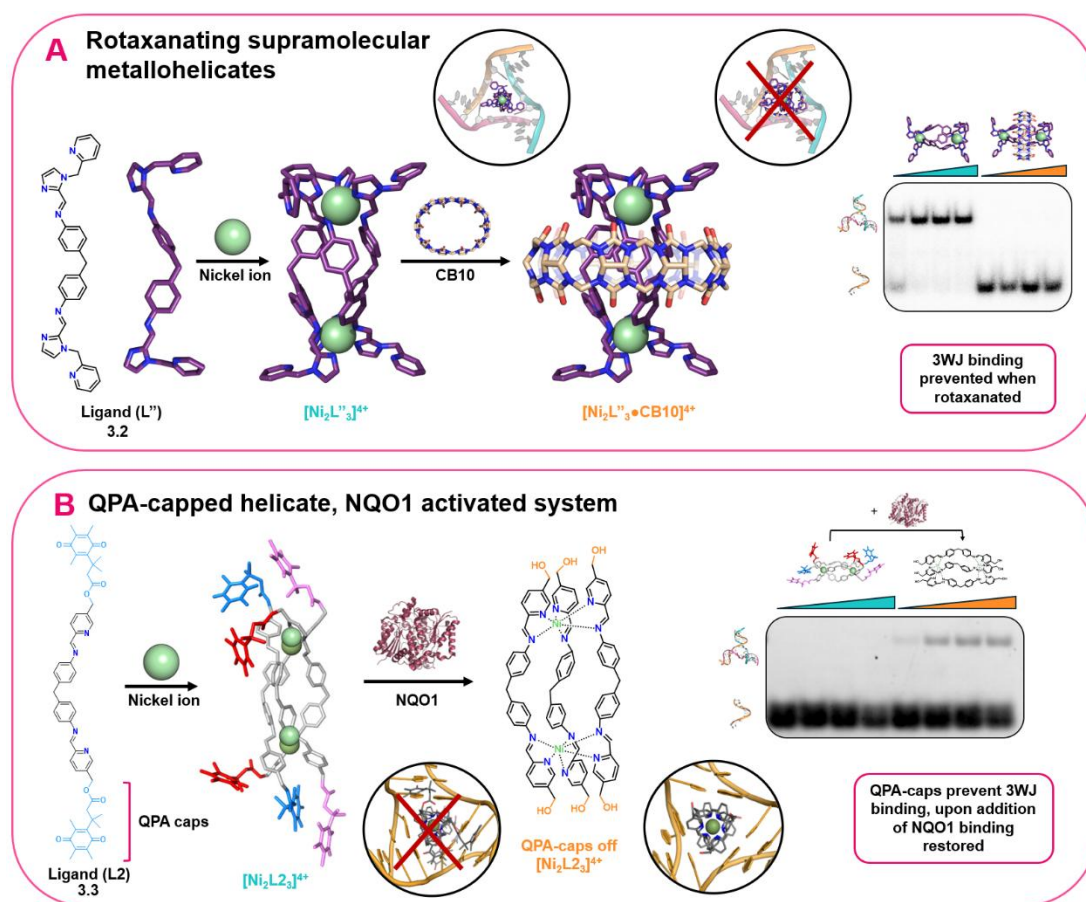


Figure 3.3: **A.** Switch on system of a supramolecular metallohelicate encased by rotaxane, preventing three-way junction binding until released, **B.** NQO1 activated system, helicate three-way junction binding controlled by QPA-caps

Continuing the search for a “switch on” system, Hannon *et al.* investigated the assistance of an enzyme, NQO1, in the activation of a pro-junction binder.⁴²³ A trimethyl-substituted quinone bound to β -dimethyl-substituted propionic acid (QPA, **3.3**) (**Figure 3.3B**)⁴²⁴ has previously been used in NQO1-activated delivery systems^{425–428}, and prodrugs.^{429–432} The NQO1 enzyme reduces the quinone, which is followed by a lactone ring closure that releases the compound.^{433,434} QPA caps were placed on all six exposed ends of a helicate, which could successfully be removed by NQO1 (and co-factor NADPH), as seen by mass spectrometry. Looking into 3WJ binding properties, no stabilisation of a 3WJ structure is seen in the presence of capped-helicate in a gel electrophoresis experiment, however upon addition of NQO1, a 3WJ band appears. To eliminate the possibility of a different mechanism occurring, the experiment was repeated in the presence of NQO1 as well as known NQO1 inhibitor, which as expected showed no 3WJ band, confirming the mechanism of action. Further confirmation was acquired through melting experiments, with no temperature increase measured for the denaturing of the 3WJ-DNA in the presence of the capped helicate. In contrast, an 18 °C increase is calculated with the uncapped helicate. Finally, a cell viability assay showed an $IC_{50} = 3 \mu M$ value for a capped helicate, presumably uncapped in the cell by NQO1, whereas a 5-fold decrease was measured in the presence of the NQO1 inhibitor. The results discussed indicate the synthesis of a successful pro-junction binder, activated upon internalisation by the cell.⁴²³

Recently, the originally designed Ni(II) cylinders were investigated through a series of *in vitro* assays, further analysing their affinity and selectivity for 3WJs.⁴²² A competitive FRET-melting assay and gel electrophoresis assay showed good selectivity for symmetrical 3WJs in the presence of dsDNA or G-quadruplex DNA. A DNA polymerisation arrest assay suggested the cylinders can inhibit DNA synthesis by binding to the junctions. Additionally, moderate cytotoxicity was measured, mainly associated with DNA damage. Despite their 3WJ selectivity, the cylinders also bind to G-quadruplexes, and often G4 binders are cytotoxic via a DNA damaging mechanism by G4-mediated TOP2 trapping. However, upon co-treatment with a topoisomerase II inhibitor, no such mechanism was observed for the cylinders, suggesting their cytotoxicity may be related to their 3WJ binding properties. Finally, upon co-treatment with DNA damage response inhibitors, moderate synergism was recorded.⁴²²

3.1.3 Peptide helicates

Other modifications that have been made to helicates include the introduction of arginine residues, onto the ends of the cylinder ligands (**Figure 3.4A**).⁴³⁵

Enantiomerically pure arginines were attached to the parent ligand (**3.4** and **3.5**), controlling the chirality of the cylinder. A PAGE experiment assessing the ligands 3WJ binding properties revealed improved binding from the arginine-bound ligands compare to the original helicates.

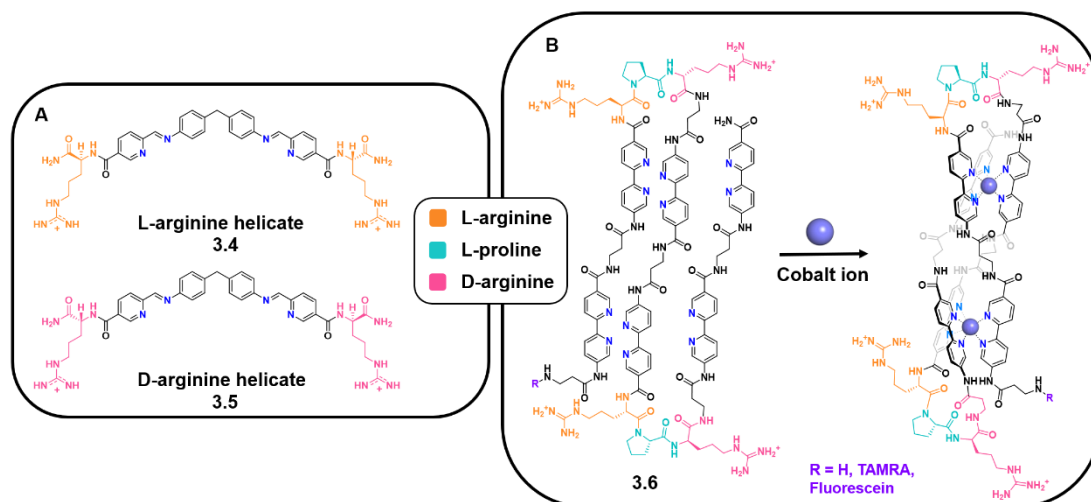


Figure 3.4: **A.** Helicates bearing L or D arginine groups at either end, improving their three-way junction binding, **B.** Cobalt helicate formed by three ligands linked via a short peptide

An alternative hypothesis focused on improving helicate stabilisation and folding by tethering three ligands together with the help of a short peptide (**3.6**) attached at both ends (**Figure 3.4B**).⁴³⁶ The chirality of the amino acids used was chosen carefully to induce a reverse β -turn, aiming to trigger the desired folding of the peptide, yielding iron and cobalt coordinated helicates. Due to certain limitations concerning the self-assembly of the helicates, the Co^{II} helicate was oxidised to its Co^{III} analogue, significantly increasing its stability. However, the Fe^{III} analogue performed better than both, with higher affinity and selectivity for 3WJs. A fluorescently tagged version of the helicate was imaged, partially matching its distribution in the nucleus with the localisation of DNA replication sites. This suggested that the helicate can target real time 3WJs forming during DNA replication in functional cells, the first demonstration of 3WJ staining with a fluorescent probe.⁴³⁶

Working towards 3WJ targeting compounds that can be translated to anticancer agents, in 2023, Vasquez Lopez *et al.* disclosed the design of a chemical nuclease able to selectively target and cleave 3WJs in vitro and in mammalian cells.⁴³⁷ A Co^{II} version of the peptide helicate described above was chosen (**Figure 3.4B**), displaying

good 3WJ recognition properties *in vitro*, confirmed by fluorescence titration experiments. Gel electrophoresis confirmed 3WJ binding, showing good 3WJ selectivity in the presence of dsDNA competitor. A PAGE assay using three labelled oligonucleotides indicates the ability of the chemical nuclease to selectively cleave 3WJ structures, even in the presence of canonical DNA. A fluorescently-labelled version of one of the peptide enantiomers was also synthesised and used to monitor the peptide live in cells. It was observed that it localised mainly at DNA replication sites, with minimal off-target RNA binding. The cleaving activity of the peptide helicate was also assessed in cells through a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. This experiment is a method of detecting DNA fragmentation, the TUNEL signal increases as the number of free DNA ends increase. As a result, the TUNEL signal increase observed upon addition of the peptide helicate appears to correspond to its cleavage of 3WJ sites, exposing more free DNA ends.

Another approach to peptide helicates investigated a different family of compounds that display the desired C_3 symmetry, namely the benzene-1,3,5-tricarboxamides (BTAs).⁴³⁸ BTAs have been used by Meijer in synthesising supramolecular helical aggregates stabilised by π -stacking and intramolecular hydrogen bonds. An attractive feature of these compounds was discovered when they were found to form supramolecular helical polymers in water from BTA-bearing dipeptides. This gave rise to the vision of a metal-chelating C_3 -symmetric tripeptide bearing a central aromatic unit, able to self-assemble into chiral supramolecular helical polymers and bind to 3WJs. BTMA-1 monomers (**Figure 3.5**) were found able to fold into discrete helicates upon coordination with Co^{II} ions, displaying high affinity and selectivity for 3WJs in gel electrophoresis and fluorescence quenching assays.

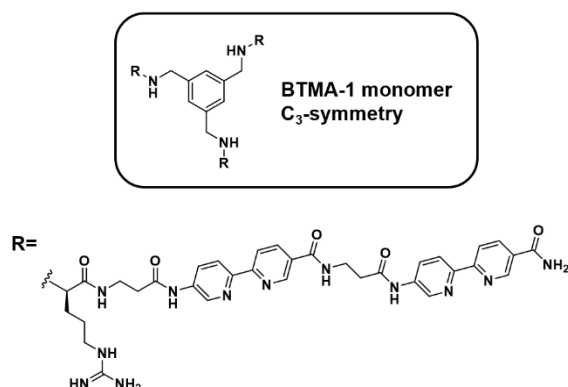


Figure 3.5: BTMA-1 monomer

Another approach assessed the addition of a bipyridyl ligand on the *N*-terminus of bacteriophage T4 Fibrin foldon (T4Ff) (**3.7**) to the helicate (**Figure 3.6**). T4Ff is a 27-

amino acid peptide that self-assembles into a trimeric propeller-like structure, envisioned to act as an appropriate support for helicate formation by ligand arrangement. Results indicate that despite the desired ligand arrangement occurring, Fe^{II} ions are still required for the helicate to bind to a 3WJ, which it does selectively.⁴³⁹

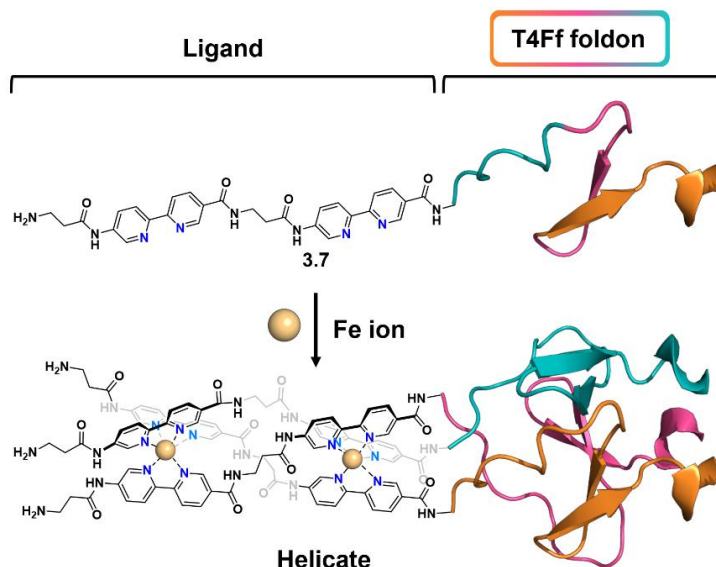


Figure 3.6: Helicate modification by the addition of bacteriophage T4 Fibrin foldon at one end of the ligand

In summary, the work on this group of compounds has led to the understanding of key features required for 3WJ binders that target the cavity at the junction cross-over point. The initial research concerning helicates targeting the 3WJ paved the way to a new and exciting field of research.

3.1.4 Triazacyclononane derivatives

Monchaud and co-workers designed a series of biophysical assays to accurately evaluate 3WJ binding affinity and selectivity of multiple candidates in a timely manner.³⁹⁹ UV-melting and FRET-based melting assays were used to assess stabilisation of DNA upon binding to a compound, comparing the effects on dsDNA and 3WJ. A displacement assay utilising a 3WJ branching point binding dye was also used to assess 3WJ affinity and selectivity. To confirm candidates were binding at the branching point, the assay was repeated with a dsDNA-binding dye, which would only be displaced if the compound chose to bind in the small duplex arms of the junction. A series of compounds were designed, maintaining the C_3 -symmetrical spatial arrangement of Hannon's helicates, using 1,4,7-triazacyclononane (TACN, **3.8**) as a scaffold. A TACN-quinoline compound (TACN-Q, **Figure 3.7**), previously studied as a fluorescent zinc sensor⁴⁴⁰, was chosen as it forms a helical trimeric structure upon arrangement around a metal ion. The compound was carried through the designed

assays and was identified as promising candidate, with moderate 3WJ stabilisation ($\Delta T_m = 4.9\text{ }^{\circ}\text{C}$, $1\text{ }\mu\text{M}$) affinity but notably high selectivity for 3WJ over other DNA structures, showing no stabilisation. Modifications were made to the structure to pin down the significance of the structural elements, the quinolines were replaced with naphthalenes (TACN-N, **3.10**) and acridines (TACN-A, **3.9**), demonstrating lower stabilising effects. The structure was also chelated to different metal ions, which showed that the metal-free TACN-Q was the best 3WJ binding compound.

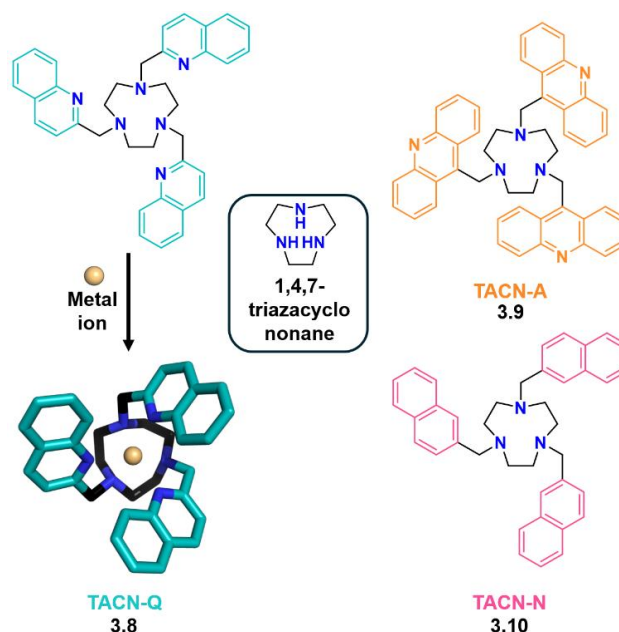


Figure 3.7: Structure of three-way junction binding, metal-chelating, agent 1,4,7-triazacyclononane quinoline (TACN-Q), and two variations assessing quinoline importance explored through replacement with acridine (TACN-A) and naphthalene (TACN-N)

3.1.5 Triptycene derivatives

Another class of compounds that gained interest as potential 3WJ binders are triptycene derivatives. Triptycene holds a threefold symmetric architecture, seemingly important when targeting 3WJs, and makes up an ideal size for the branching point hole in the middle of the junction (**Figure 3.8**). Derivatives **3.11**, **3.12** and **3.13** were assessed initially, displaying significant stabilising effects of a 3WJ when tested in a melting experiment, indicating selectivity as there were no signs of dsDNA stabilisation.⁴⁴¹ To further assess 3WJ binding, a displacement assay was carried out, where the 3WJ-folding strand was bound to a complimentary ssDNA molecule, preventing junction formation. The triptycene compounds were able to compete with the complimentary strand, fold the DNA into a 3WJ and stabilise it, confirmed both through a fluorescence and a gel shift assay. A new class of triptycene compounds bearing amino acid side chains, **3.14**, **3.15** and **3.16**, were designed and tested against a DNA trinucleotide repeat, (CAG) $\cdot$ (CTG), expansions of which have been

associated with several neurodegenerative diseases.²²¹ The displacement assay was repeated with sequences only made up of these repeats, and the compounds were once again competing with the complimentary strand, showing good affinity.^{221,442}

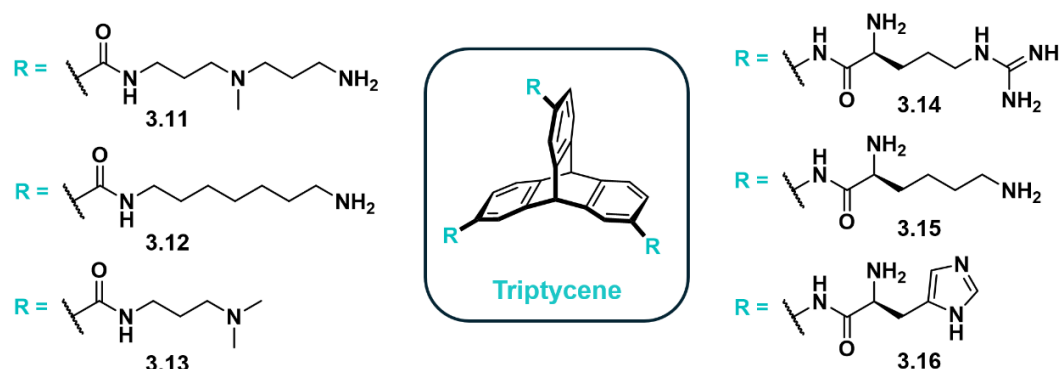


Figure 3.8: Triptycene compounds assessed for their three-way junction binding abilities through variation of the sidechains

3.1.6 Azacryptands and azacyclophanes

Shortly after investigating triazacylononanes, Monchaud and co-workers turned to a new class of compounds called azacryptands, previously investigated as G-quadruplex binders.⁴⁴³ A few promising features drew their attention, namely their cylinder-like shape seemingly capable of fitting in the central junction cavity, their charges to participate in electrostatic interactions with the junction, and finally their flexible aromatic group, able to freely rotate and position themselves in orientations encouraging π -stacking interactions with base pairs lining the walls of the cavity. A summary of the investigated azacryptands is shown in **Figure 3.9**. The aromatic groups are linked via the same linkers, allowing variation across the stacked aromatic complexes. A FRET-melting assay measured good stability of a 3WJ upon addition of the ligands ($\Delta T_m = 7 - 15$ °C, 1 μ M), insignificantly affected by the addition of an unlabelled dsDNA competitor. However, unsurprisingly due to their previous quadruplex binding investigations, the addition of unlabelled quadruplex DNA significantly decreased the 3WJ stabilisation. To explore the compounds' ability to interact with 3WJs, a PAGE assay was carried out, evaluating their ability to assemble 3WJs from a mixture of three partially complimentary DNA strands. The results highlighted the importance of assessing a ligand's 3WJ binding potential by both FRET-melting and EMSA assays, as some candidates performed well in both, but certain candidates only displayed promising results in one. CD experiments were carried out for azacryptand candidate 2,7-TrisNP (**3.18**), suggesting the ligand binds in the central cavity of the junction, as intended. Finally, antiproliferative properties of

the compounds were assessed in melanoma cancer cells B16, indicating some good toxicity.

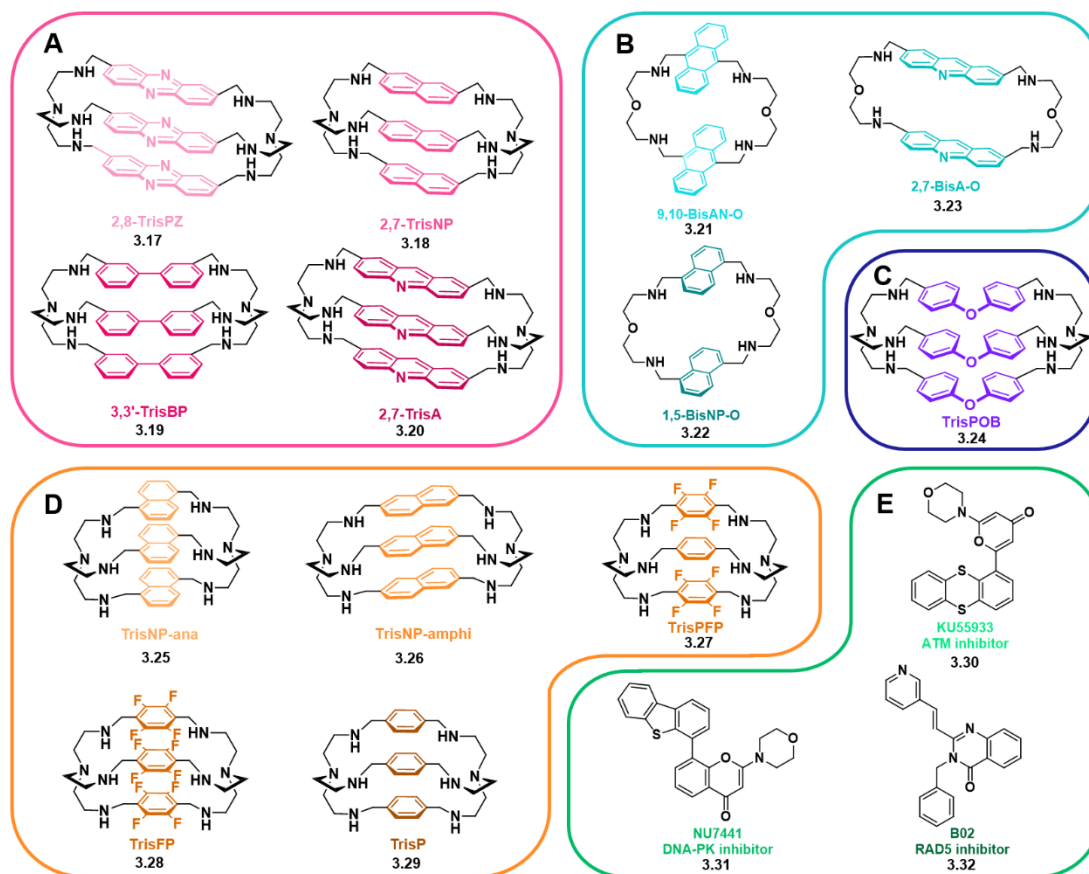


Figure 3.9: **A.** Initial azacryptand structures investigated for their three-way junction binding abilities, due to their three-fold symmetry and previous success in interacting with other DNA structures (G-Quadruplexes), **B.** Promising macrocycles identified through a high-throughput three-way junction screen, **C.** Azacryptand further explored due to its promising three-way junction binding abilities and cell-study performance, **D.** Recent optimised azacryptands highlighted due to their high performance upon assessment via a series of assays, **E.** DNA repair response inhibitors used to investigate synergism with TrisPOB

Development of a high-throughput isothermal three-way junction screening assay (3WJ-screen) allowed for assessment of more azacryptand-like derivatives.⁴⁴⁴ The assay monitored the ligands' abilities to shift the equilibrium between the three separate DNA strands and the folded three-way junction state. Two out of the three oligonucleotides are labelled with a FRET fluorophore pair, FAM and TAMRA, FAM emission is measured in the absence and presence of a ligand, a decrease indicating the strands are sitting in a folded 3WJ state due to the ligand binding, bringing the fluorophores close. The selectivity of the compounds can be assessed on the same plate by the addition of unlabelled competitors. An initial screen of 25 candidates revealed two new promising azacryptand-like macrocycles, 9,10-BisAN-O (**3.21**) and 1,5-BisNP-O (**3.22**), previously studied for their binding properties with G-quadruplexes.

In a later study, 1200 candidates were tested in an improved version of the 3WJ-screen, where three candidates significantly stood out, two azacyclophanes, 1,5-BisNP-O (**3.22**) and 2,7-BisA-O (**3.23**), and an azacryptand, 3,3'-TrisBP (**3.19**). The compounds were tested in a set of preliminary cell-based assays.^{403,444} All three compounds were found to have antiproliferative properties in two breast cancer cells. Unfortunately, the azacyclophanes were found to be even more toxic in normal fibroblasts, but the azacryptand displayed a 2-fold decrease in toxicity.

Due to 3,3'-TrisPOB's (**3.24**) success, three novel azacryptands were synthesised.⁴⁴⁵ The compounds were carried through the assays mentioned above, displaying very good selectivity for 3WJs. A cell-based assay measuring cytotoxicity in MCF7 breast cancer cells measured sub micromolar IC₅₀ values. Following up from an immunodetection study indicating that the majority of the cytotoxicity can be attributed to the compound's DNA damaging effects, a synergy assay investigating chemically induced synthetic lethality was carried out. Three inhibitors were chosen, KU55933 (**3.30**), NU7441 (**3.31**) and B02 (**3.32**), acting on different DNA repair pathways, TrisPOB (**3.24**) showed great promise, displaying synergistic interactions with all three DNA repair inhibitors, suggesting a new potential combinatorial therapy focusing on selectively targeting DNA structures.⁴⁴⁵

TrisPOB (**3.24**) and 2,7-TrisNP (**3.18**) were also assessed further due to their dual targeting ability of G-quadruplexes and much higher preference for 3WJs.⁴⁰¹ Both azacryptands showed synergistic effects with the previously mentioned DNA repair response inhibitors. A second synergy assay with topoisomerase II inhibitors led to the conclusion that their cellular activity is more likely associated with their 3WJ binding abilities rather than G-quadruplex.

In a recent structural optimisation study, thirty-two azacryptands were evaluated to identify candidates with improved 3WJ binding properties.⁴⁰² Affinity for 3WJs was tested via FRET-melting assay comparing the candidates' stabilising effects on 3WJs, dsDNA and G-quadruplex DNA. Analysis of the results pointed to five promising compounds that highly stabilise the 3WJ, three of which maintain 3WJ selectivity in the presence of the alternative DNA structures. All five candidates were assessed in a 3WJ screen assay to observe their ability to promote the folding of a 3WJ. Two of the three strands were labelled with a fluorophore (TAMRA) and a quencher (BHQ2), enabling monitoring of 3WJ folding upon binding to a ligand due to FRET, which highlighted four good candidates. A new assay was described, investigating whether ligand-stabilised 3WJs will interfere with DNA processes *in vitro*. Drawing inspiration

from a G4 stabilising assay⁴⁴⁶, a modified qPCR stop assay was designed, involving the amplification of 3WJ-containing 64-bp strand of DNA by Taq DNA polymerase, following a commonly used qPCR procedure. The resulting amplification obtained is directly linked to DNA polymerase inhibition caused by ligand stabilisation of the 3WJ in the sequence, therefore can be quantified. This assay supports the hypothesis in which 3WJ stabilisation can act as a physical barrier to DNA polymerase activity.

In summary, azacryptand derivatives show promise as 3WJ binding compounds and have provided the field with insights regarding the potential cellular effects of 3WJ binders.

3.1.7 Calix[3]carbazole

Cationic calix[3]carbazole (**3.33**) was investigated as a 3WJ binding compound as it holds the threefold symmetry characteristic (**Figure 3.10**) of previously described 3WJ binders.⁴⁴⁷ Additionally, its structure resembles a C_3 symmetric structure, similar to the central cavity of the junction. Due to its pre-organised conformation, it was expected not to interact with the major/minor grooves of the junction duplexes nor intercalate with base pairs, therefore increasing its selectivity for 3WJs. The compound is characterised by a very useful fluorescence feature, as upon binding to DNA the intrinsic fluorescence of the compound increases, allowing binding monitoring through fluorescence titration experiments. UV melting was carried out to measure the stabilising effects of the compound ($\Delta T_m = 12\text{ }^{\circ}\text{C}$, $1.5\text{ }\mu\text{M}$), and CD experiments, ^1H NMR spectroscopy and a PAGE assay were performed to confirm 3WJ binding. UV titrations indicated non-specific binding with dsDNA via electrostatic interactions, rendering the need for higher 3WJ selectivity to be used as a 3WJ fluorescent probe.

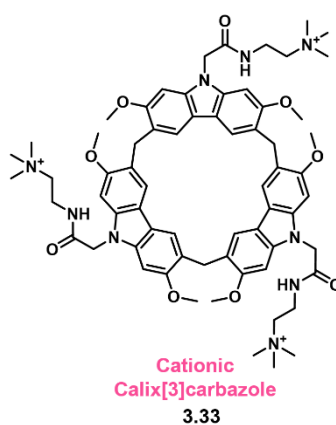


Figure 3.10: Structure of cationic Calix[3]carbazole

3.1.8 Tri-peptide

In 2022, Vazquez and co-workers described a tri-peptide structure for the recognition of A-T rich 3WJs, designing the first compound aimed to target the junction's duplex arms.⁴⁴⁸ Due to the lack of defined structural elements of the central cavity of 3WJs, the tripeptide aimed to mimic the binding mode of certain natural proteins that selectively recognise 3WJs from the duplex arms.⁴⁴⁹ The structure was finalised through molecular dynamics simulations, with a 1,3,5-tristyrylbenzene centre (**3.34**) modified at the ends with a linker bound to a known A-T hook peptide (**Figure 3.11**).⁴⁵⁰

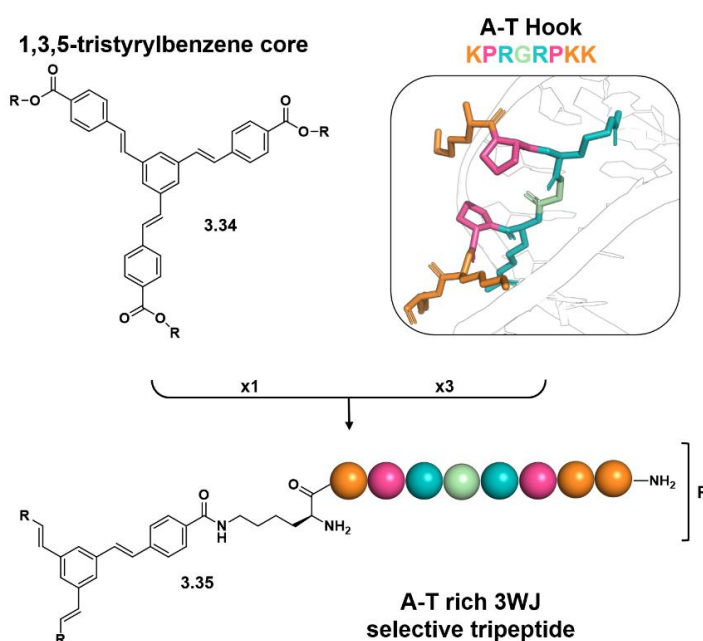


Figure 3.11: A-T hook tripeptide designed with a 1,3,5-tribromobenzene core, extending out through an A-T hook, displaying selectivity towards A-T rich three-way junctions

The central core is intrinsically fluorescent therefore binding was assessed through fluorescence titration as an increase in fluorescence occurs upon binding to DNA, which showed selective binding to an AT-rich 3WJ, and no binding to a GC-rich 3WJ or dsDNA. The results were also confirmed through CD experiments and gel electrophoresis.

3.1.9 Conclusions

Motivation for the study described in this chapter was inspired by the three-way junction binding compounds described in this section, as it became clear that certain factors have to be taken into consideration when designing 3WJ binders. For example, the three-fold symmetry of a compound is crucial, and specific dimensions should be considered if targeting the central cavity. However, when thinking about the structure of a three-way junction, the centre of the junction, where the three DNA

strands are intertwined, is not structurally consistent. The lack of defined structural elements makes it hard to target selectively, therefore, targeting the arms of the junction was of more interest. The junction arms are small sections of duplex DNA, consequently the final compound must include a strong dsDNA binding element whilst simultaneously being selective for three-way junctions. To avoid targeting other DNA structures similar in shape, including Y forks, one approach could involve targeting all three dsDNA sections with one compound at the same time.

3.2 Chapter aims

This chapter describes the design and synthesis of two novel acridine-based trimeric small molecules targeting 3WJs. The design of the structures aims to incorporate key elements required for 3WJ binding, considering the three-way binding compounds described in **section 3.1**, in combination with the DNA intercalating unit 9-aminoacridine-4-carboxamide. The aim is the synthesis of compounds able to selectively bind and stabilise three-way junctions over other DNA structures. Beyond small molecule synthesis, this chapter investigates the development of biophysical assays to investigate DNA binding.

3.3 Results and discussion

3.3.1 Design of the trimers

As discussed in **section 1.5.2**, 9-aminoacridine-4-carboxamide binds well to dsDNA. Additionally, the FRET melting data recorded for the acridine dimers discussed in **section 2.3.15**, confirmed that two acridine moieties could stabilise DNA forms better than one. Therefore, the question arose of whether three would provide even better binding, binding to each arm of the junction. The compound designed would have to direct the acridine groups towards the desired DNA binding locations. This would require a degree of rigidity when directing the acridine groups as well as some flexibility to allow for intercalation.

Lengkeek *et al.*, described the synthesis of a series of potential precursors for DNA intercalators.⁴⁵¹ Drawing inspiration from known DNA intercalators, the paper disclosed several styryl compounds bearing a central benzene core. One of the compounds presented was of triangular shape, made up of a benzene ring in the centre, symmetrically extending out of ring positions 1, 3 and 5 via an alkene further bound to another benzene ring, as seen in **Figure 3.12**.

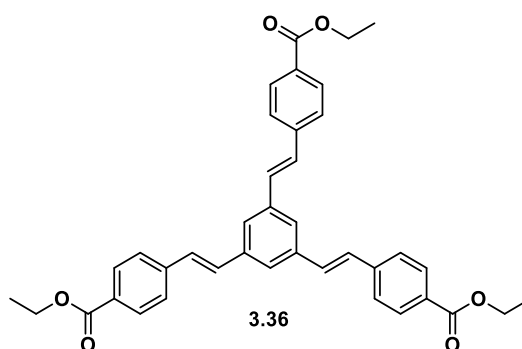


Figure 3.12: Structure of 1,3,5-tristyrylbenzene

This structure was thought to accurately mimic the shape of a three-way junction and could be used as the central unit for the final compound, attaching the acridines at the ends of the three extensions. The 1,3,5-tristyrylbenzene core would be expected to rest on top of the branching point in the junction, a linker could be attached and the end of each arm extension, carrying 4-carboxamide-9-aminoacridine on the other end to intercalate with the DNA duplex branches (**Figure 3.13**).

A similar hypothesis was followed in 2022 by Vázquez *et al.* incorporating the central unit in a 3WJ targeting compound, attaching a well-studied A-T hook, nine amino acid long peptide, onto the arm extensions.⁴⁴⁸ The compound was designed to selectively target A-T rich three-way junctions, following simulation predictions.

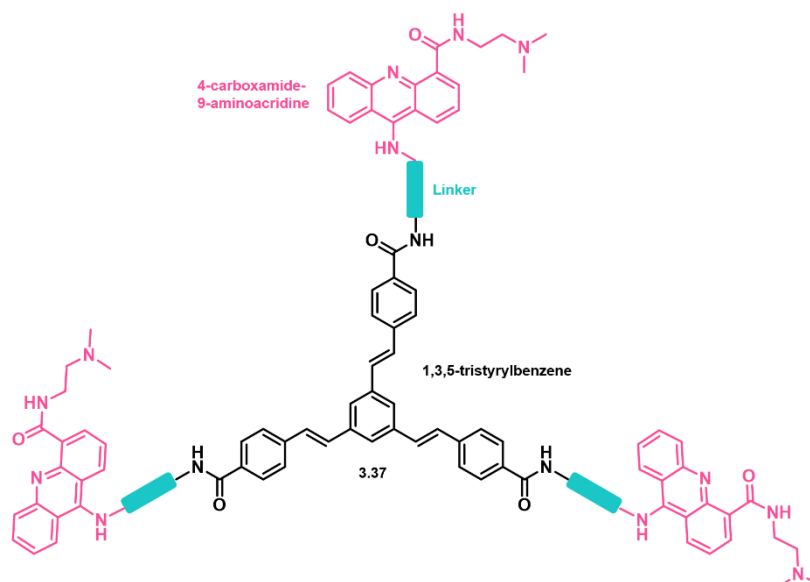
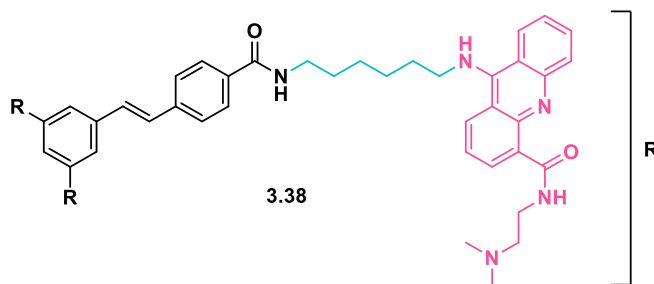


Figure 3.13: Proposed design for a three-way DNA junction targeting tri-acridine compound

The basic outline of the final design is presented in **Figure 3.13**. As discussed in **section 1.5.2**, the carboxamide chain attached to the acridine shifts the molecule to intercalate in a specific orientation. Additionally, the design aimed to allow the acridine groups the flexibility to correctly align, therefore, a linear carbon chain was chosen as

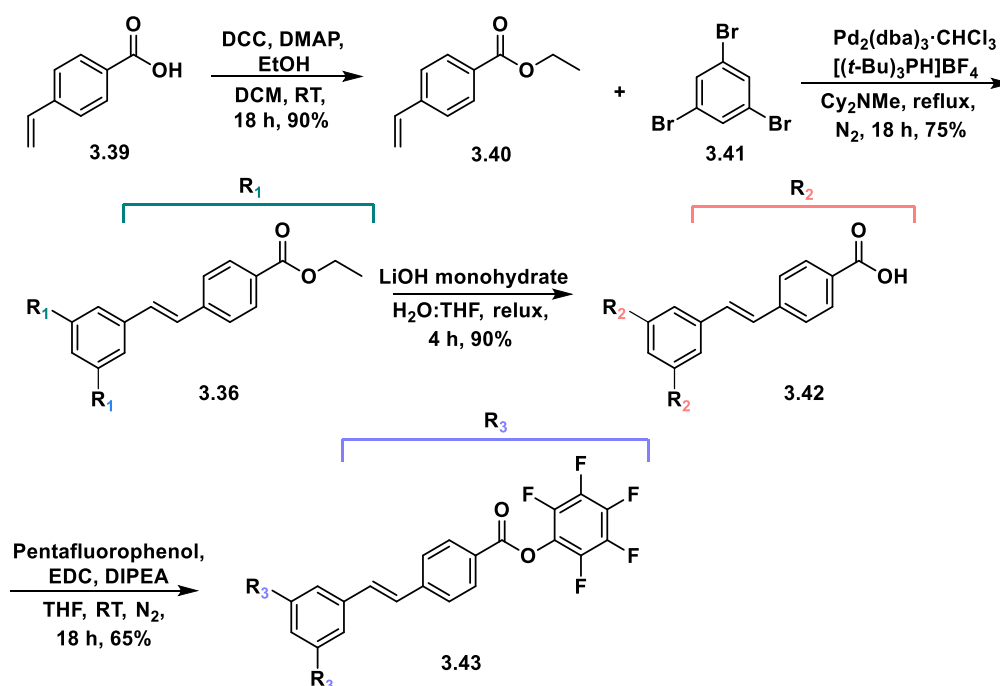
the linker, mimicking the length of the selected linker from Vázquez *et al.* The final design (**3.37**) is made of a 1,3,5-tristyrylbenzene core central unit, linked to three 9-aminoacridine-4-carboxamide groups via a six-carbon linker (**Scheme 3.1**: Final design of trimeric acridine structure).



Scheme 3.1: Final design of trimeric acridine structure

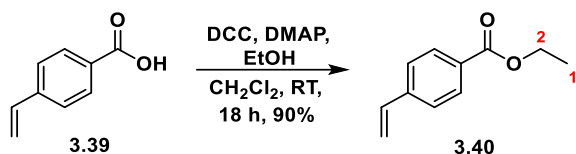
3.3.2 Synthesis of six-carbon linked acridine trimer

The synthesis of the trimeric compound was divided into two sections, the synthesis of the central unit, and the synthesis of the linker-bearing acridine. The synthesis of the middle unit is summarised in **Scheme 3.2**. Commercially available 4-vinylbenzoic acid (**3.39**) was converted to 4-vinylbenzoate (**3.40**) before carrying out a Heck cross-coupling reaction with 1,3,5-tribromobenzene (**3.41**). The resulting tri-ester (**3.36**) was converted to the relative tri-acid (**3.42**) via hydrolysis, and lastly pentafluorophenol was attached at each end to act as a good leaving group prior to coupling to the acridine (**3.43**).



Scheme 3.2: Synthetic pathway designed for the synthesis of the central unit of the acridine trimer

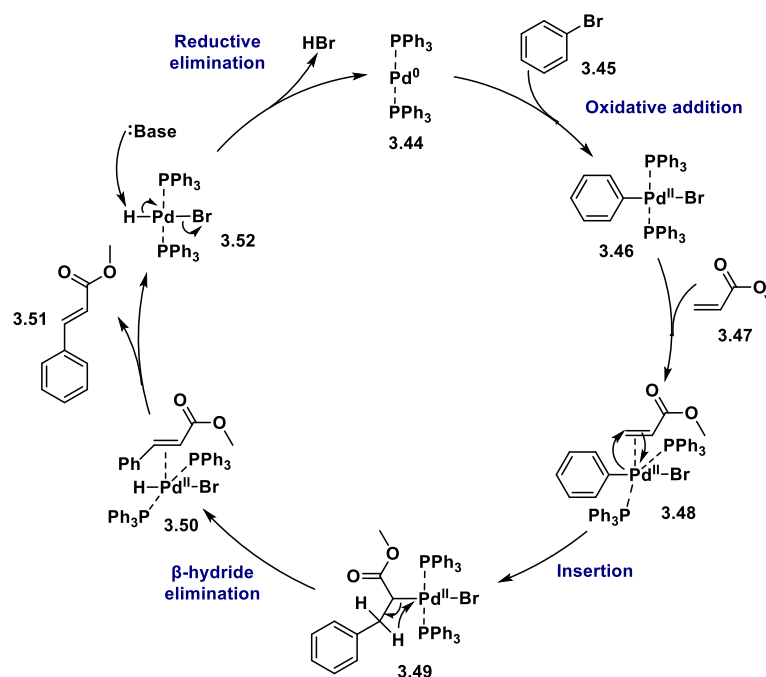
Following a procedure published by Gao *et al.*, commercially available 4-vinylbenzoic acid was stirred with ethanol and coupling reagents DCC and DMAP overnight to yield ethyl 4-vinylbenzoate in a 90% yield.⁴⁵² The sample was stored in the freezer as it was found to degrade over time if left out at room temperature.



Scheme 3.3: Esterification of 4-vinylbenzoic acid with coupling agents DCC and DMAP

The formation of the compound was confirmed by the presence of two new proton environments in the ^1H NMR spectrum, corresponding to the ethyl group addition. A quartet at 4.37 ppm corresponding to protons carbon 2, integrating to two, and a triplet at 1.39 ppm corresponding to protons bound to carbon 1, integrating to three (**Scheme 3.3**).

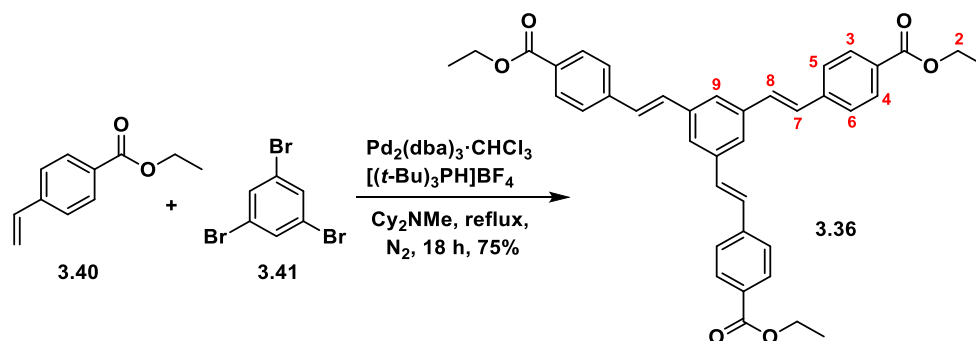
The next step involved the coupling of three **3.40** molecules onto a tri-bromo benzene ring, completed through a Heck cross-coupling reaction. A Heck reaction is a palladium-catalysed coupling between an aryl halide and alkene in the presence of a base, forming new carbon-carbon bond.⁴⁵¹ Heck reactions selectively produce the *trans* isomer as it is more stable than the *cis* and less sterically hindered.



Scheme 3.4: Heck cross-coupling mechanism, beginning with an oxidative addition of the palladium catalyst (3.44) by the aryl halide (3.45), followed by insertion of the olefin (3.47) into the Pd-Ph bond (3.48), β -hydride elimination yields the alkene reaction product (3.51) and regeneration of the palladium catalyst (3.44)

Commercially available 1,3,5-tribromobenzene (**3.41**) was chosen for the central ring. Following published literature by Lengkeek *et al.*, ethyl 4-vinylbenzoate (**3.40**) and 1,3,5-tribromobenzene (**3.41**) were heated under reflux in a 3 to 1 ratio, in the presence of palladium catalyst $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$, $[(t\text{-Bu})_3\text{Ph}]\text{BF}_4$ and a base, *N,N'*-dicyclohexyl methylamine.⁵¹

The reaction is carried out in tetrahydrofuran. To ensure the solvent was as dry as possible, it was degassed using the freeze-pump-thaw method prior to the addition of any reagents, a technique used to remove dissolved gasses, in this case oxygen. During this method the flask containing the solvent is placed in liquid nitrogen, completely freezing the contents, the frozen solvent is then allowed to thaw under vacuum. During this process the headspace above the frozen solvent is removed as well as any dissolved gasses. This cycle is repeated a total of three time to ensure the majority of the oxygen has been removed.

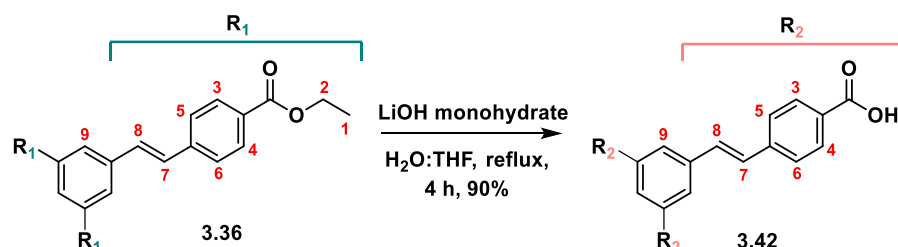


Scheme 3.5: Heck cross-coupling reaction between 1,3,5-tribromobenzene (**3.41**) and 4-vinylbenzoate (**3.40**)

Once the reaction is complete, the palladium is removed via filtration and the product is further purified by column chromatography. The final product is an off-white powder successfully isolated with a 75 % yield.

In the ^1H spectrum of **3.36**, the alkene proton peaks 7 and 8, shift from 6.75 ppm, 5.86 ppm and 5.38 ppm (compound **3.40**) to 7.52 ppm and 7.47 ppm. The two terminal alkene protons of compound **3.40** appear as different signals, as they are not chemically equivalent due to the rotation restriction by the double bond, upon coupling they appear as doublets. Additionally, a new peak appeared at 7.89 ppm, a singlet integrating to three, which correlates to the three protons located on the central benzene ring, protons 9, further confirms the coupling was successful. The high-resolution mass spectrum showed a signal at 601.285, matching the $[\text{M}+\text{H}]^+$ ion 601.2590.

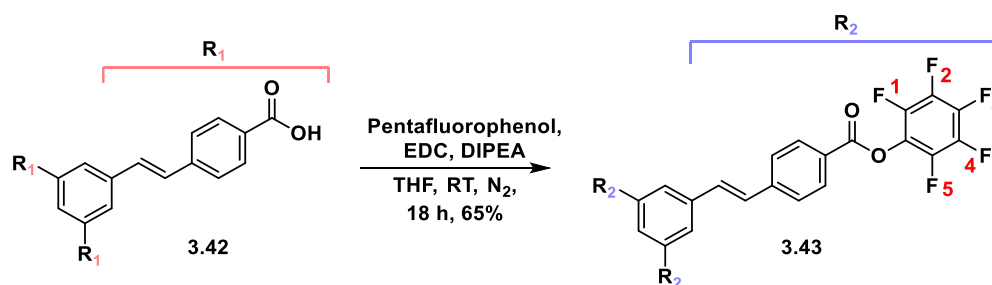
Next, the esters located at the end of the arm extensions were hydrolysed under basic conditions to yield carboxylic acids, via a saponification procedure described by Lengkeek *et al.*⁴⁵¹ Compound **3.36** was hydrolysed in the presence of LiOH monohydrate, converting the esters into carboxylate salts, which upon neutralisation with acid yield carboxylic acids, with a 90% yield.



Scheme 3.6: Hydrolysis of tri-ester **3.36** yielding carboxylic acids (**3.42**) at all three ends

The absence of the quartet and the triplet from the ^1H NMR spectrum of **3.42**, corresponding to protons 1 and 2, confirms product formation, whilst the rest of the signals remain unchanged.

Before coupling the acridine compound to the central unit, pentafluorophenol was introduced as a good leaving group to aid the reaction. Following a procedure by Vázquez *et al.*, pentafluorophenol was added to the central unit in the presence of coupling reagents. The product was isolated via filtration followed by column chromatography.⁴⁴⁸

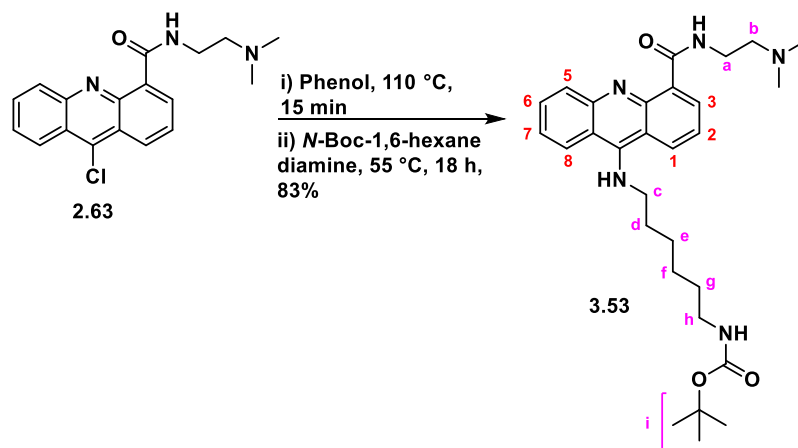


Scheme 3.7: Addition of a pentafluorophenol group at all three carboxylic acids on **3.42**

The ^1H NMR spectrum of **3.43** holds similarities to the starting material's **3.42** spectrum, with the peaks shifting slightly downfield, however there was no change in the proton environments. Therefore, a ^{19}F NMR was carried out to confirm the addition of the pentafluorophenol group, where three signals appear, corresponding to the three fluorine environments due to symmetry, 1 and 5, 2 and 4, and 3.

Upon completion of the synthesis of the central unit, the acridine group was finalised. Following the steps described in **section 2.3.1**, 4-carboxamide-9-chloroacridine was synthesised. A linker was attached to position 9, consisting of six carbons, following

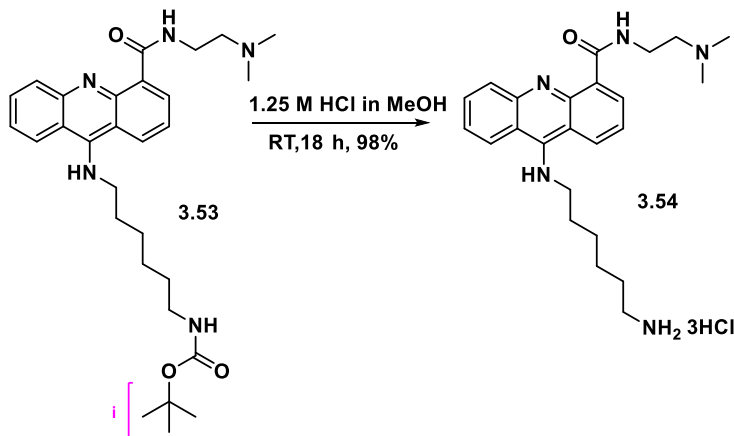
a procedure published by Howell *et al.*³⁶⁷ The central part of the trimer is rigid, therefore a carbon linker would provide flexibility to the acridines, allowing them to position themselves in the desired orientation for insertion in between base pairs when binding to DNA. The linker was added in a similar manner to the dimerization reactions described earlier, with a Boc protecting group (**scheme 3.8**) at one end to prevent binding of two acridine molecules.³⁶⁷



Scheme 3.8: Addition of *N*-Boc-1,6-hexanediamine to 9-chloroacridine-4-carboxamide

Compound (**2.63**) was stirred in phenol and heated at 110 °C for 15 minutes, forming the phenoxy intermediate before the addition of the linker, *N*-Boc-1,6-hexanediamine, allowing the solution to stir at 55 °C overnight. The product was isolated via a basic workup and column chromatography as a bright yellow oil, in a good 83% yield.

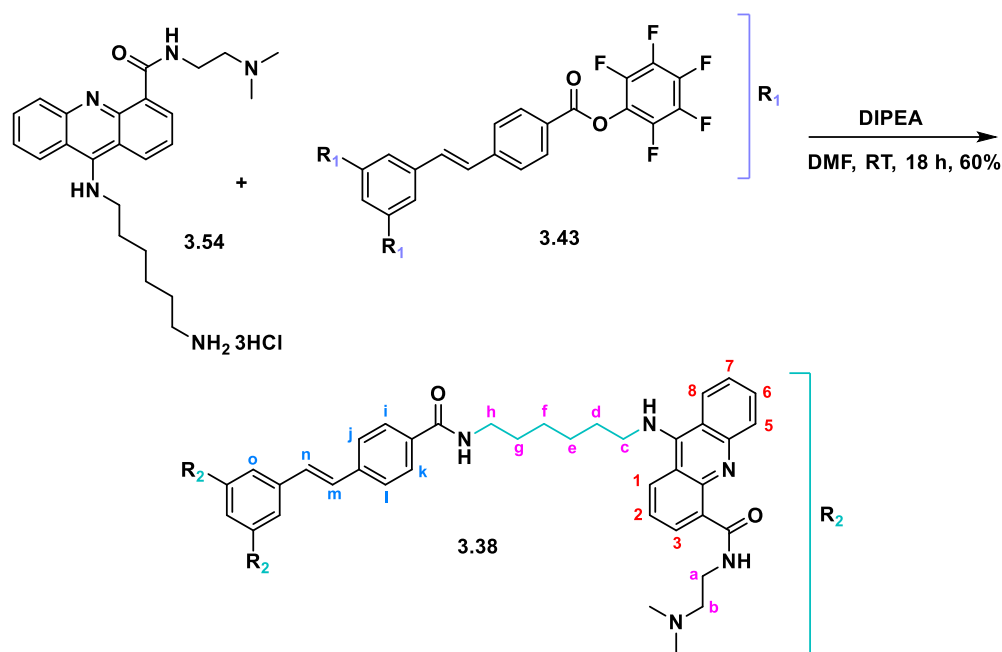
In the ¹H NMR spectrum of **3.53** (**scheme 3.8**), a sharp singlet at 1.39 ppm confirms the addition of the linker, as the signal correlates to the methyl protons *i* from the Boc protecting group, integrating to 9 protons. Additionally, six more signals are detected corresponding to the linker protons, with protons at *c* and *h* located the most downfield due to their proximity to the amine groups, and *e* and *f* the most upfield.



Scheme 3.9: Deprotection of Boc-protected acridine **3.53**

The Boc protecting group was removed by stirring the compound in 1.25 M HCl in MeOH overnight at room temperature. The compound was characterised by ^1H NMR, IR spectroscopy and mass spectrometry, matching literature.³⁶⁷ The removal of the Boc group was confirmed as there was no longer a sharp singlet correlating to the methyl protons (**scheme 3.9**), the rest of the signals remain unchanged.

With both the central unit and the substituted 9-aminacridine-4-carboxamide in hand, they could then undergo a coupling reaction. Acridine **3.54** was added in a four to one excess, to ensure all three arms would be coupled, in the presence of DIPEA. Due to the pentafluorophenol group acting as a leaving group, there was no requirement for coupling reagents. The reaction was allowed to stir overnight at room temperature in DMF. Upon completion, confirmed by TLC, the compound was precipitated out of DMF by the addition of water and purified by column chromatography. The isolated bright yellow oil was converted to a hydrochloride salt by stirring in 1.25 M HCl in MeOH for 15 minutes, followed by precipitation with EtOAc, isolating the product via filtration, and finally lyophilising the compound.



Scheme 3.10: Coupling reaction of **3.54** and **3.43** to yield the final trimer **3.38**

In the aromatic region of the ^1H NMR of **3.38** there are a total of six signals (**scheme 3.10**), integrating to forty two protons, accounting for the twenty one acridine aromatic protons, the twelve aromatic protons next to the amide, the six alkene protons as well as three protons from the middle benzene ring. The most shifted downfield signals are those corresponding to protons 1, 3 and 8. Protons 1 and 8 are shifted due to their proximity to the NH group at position 9 on the acridine. However, proton 1 is

further deshielded by the carboxamide group at position 4, shifting to 8.64 ppm appearing as a doublet integrating to three, due to its *ortho*-coupling to the proton at position 2. Protons 3 and 8 are adjacent to electron-withdrawing groups, an amine and a carbonyl, resulting in an overlap of their signals at 8.54 ppm, appearing as a multiplet, and couple to protons 2 and 7. The next signal was a doublet at 8.07 ppm integrating to three, which was assigned to proton 5 due to the deshielding effect of the nitrogen adjacent to it. A multiplet at 7.81-7.91 ppm was made up of overlapping signals, integrated to twelve protons. Within the multiplet a doublet could be observed, with the same coupling constant as a doublet at 7.71, which integrated to six. Therefore, the later signal was assigned as corresponding to aromatic protons j and l, and the six protons of the multiplet as protons i and k, which are *ortho*-coupled to one another in their respective pairs and located further downfield due to their proximity to the amide group. The third signal which was part of the multiplet is a singlet, corresponding to proton o, located on the middle ring, not coupled to any protons. Lastly, the fourth proton of the multiplet was assigned to proton 6, expected to be located further downfield than protons 7 and 2. The final signal in the aromatic region is another multiplet, integrating to twelve protons, assigned to protons 7 and 2, as well as the six alkene protons. Protons 2 and 7 are the farthest from any electron-withdrawing groups on the acridine, expected to be the most upfield.

Moving forward, a series of multiplets are located in the aliphatic region corresponding to the CH₂- groups on the linker chain as well as the carboxamide chain. A multiplet at 3.97 ppm integrating to six corresponds to protons c, which is shifted downfield due to its proximity to the amine on the acridine position 9. Next, a multiplet at 3.84 ppm corresponding to methylene group a, shifted due to the adjacent amide group and the tertiary amine. The next two signals are multiplets at 3.35 ppm and 3.24 ppm, integrating to six protons each, they were assigned to protons on carbons h and b accordingly, due to their adjacent nitrogen atoms. The methyl protons are seen as a sharp singlet at 2.83 ppm, integrating to eighteen protons. Multiplets at 1.85 and 1.51 ppm were assigned to the methylene groups at positions d and g accordingly, and finally, two overlapping multiplets at 1.28 – 1.44 ppm were assigned to protons e and f. They are the most shielded due to their distance from the surrounding electron-withdrawing groups.

The high-resolution mass spectrum showed a peak at 1685.7037, correctly matching the [M+H]⁺ ion 1685.9420.

3.3.3 Synthesis of trimer with a diethyl ether linker (3.55)

A second trimer (**3.55**) was synthesised with a diethyl ether linker (**Figure 3.14**), maintaining the primary structure of trimer (**3.38**), shortening the linker to 5 atoms instead of 6.

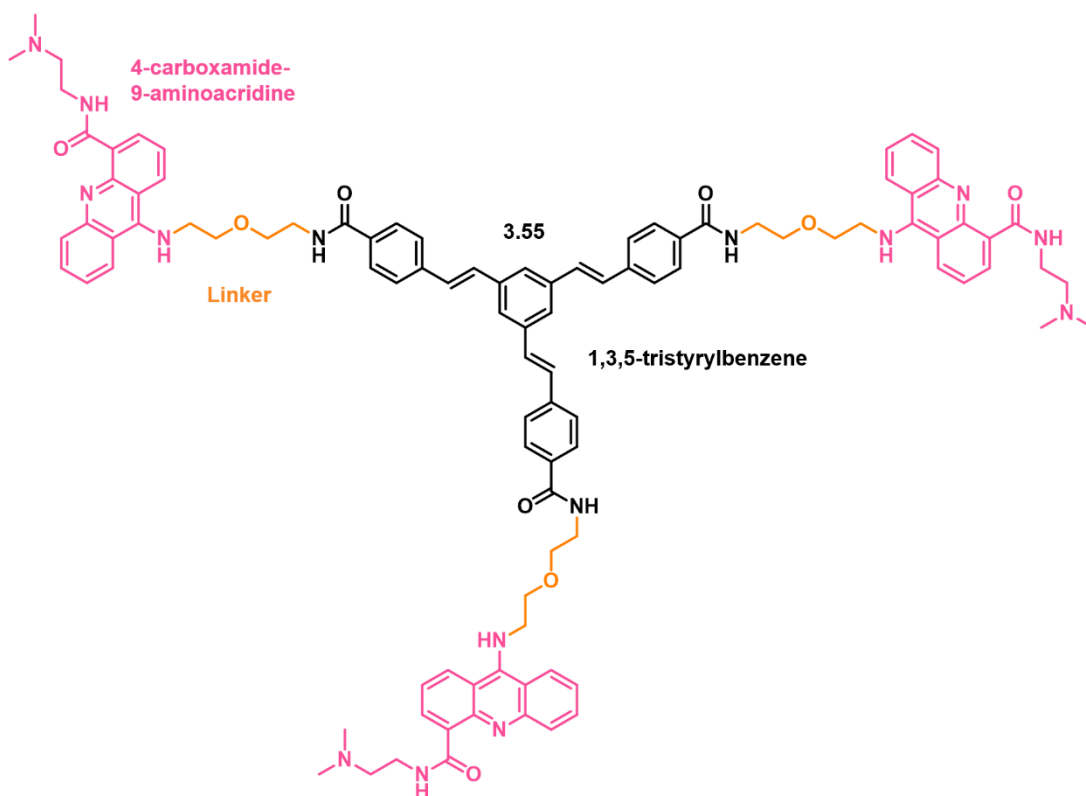
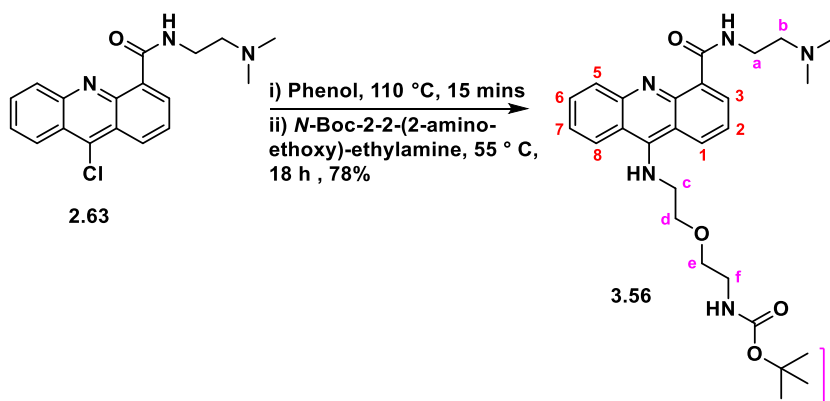


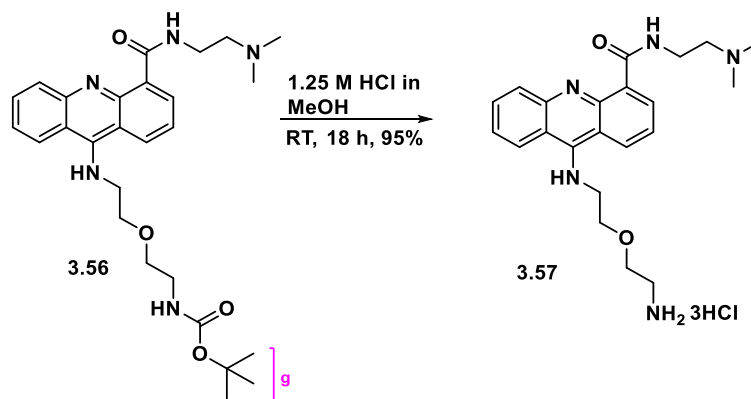
Figure 3.14: Structure of trimer **3.55** with a 5-atom linker

The central unit remained the same, therefore the new linker was coupled onto the acridine. 9-aminoacridine-4-carboxamide was coupled to *N*-Boc-2-(2-amino-ethoxy)-ethylamine by heating the acridine with phenol at 110 °C for 15 minutes, followed by cooling to 55 °C and addition of the linker. The product was isolated via workup and purification at a yield of 78%.



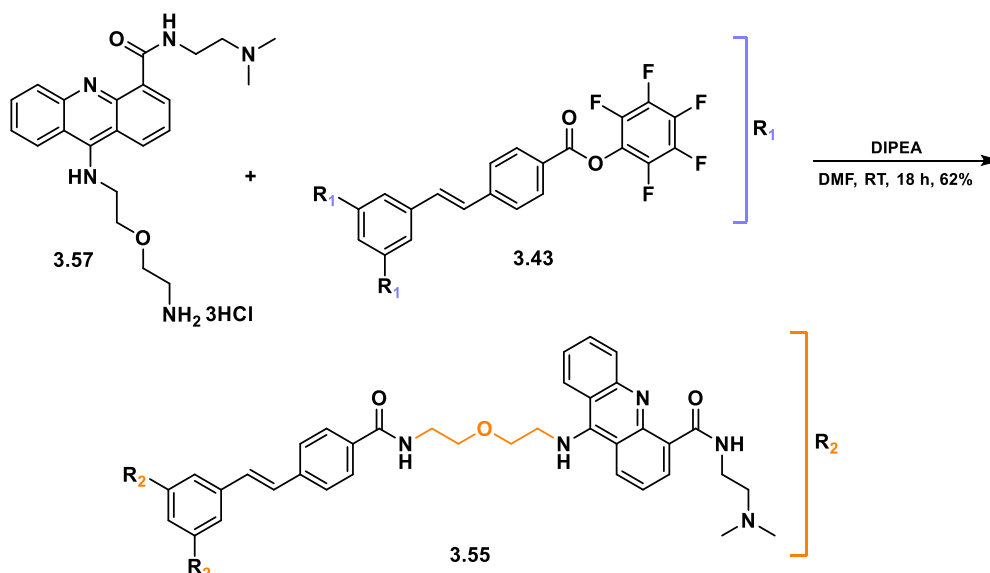
Scheme 3.11: Addition of *N*-Boc-2-(2-amino-ethoxy)-ethylamine to 9-chloroacridine-4-carboxamide

The addition of the linker was confirmed by the presence of a new signal at 1.39 ppm integrating to nine protons, corresponding to the Boc group methyl protons. An additional 4 signals were also observed in the higher aliphatic region, assigned to the new CH₂- groups added.



Scheme 3.12: Removal of Boc protecting group from **3.56**

The linker was deprotected with 1.25 M HCl in MeOH overnight at room temperature, yielding compound **3.57**, at a good yield of 95%. The compound was characterised by ¹H NMR, IR spectroscopy and mass spectrometry. The absence of the sharp singlet at 1.39 ppm corresponding to protons g (**scheme 3.12**), confirms the removal of the Boc protecting group.



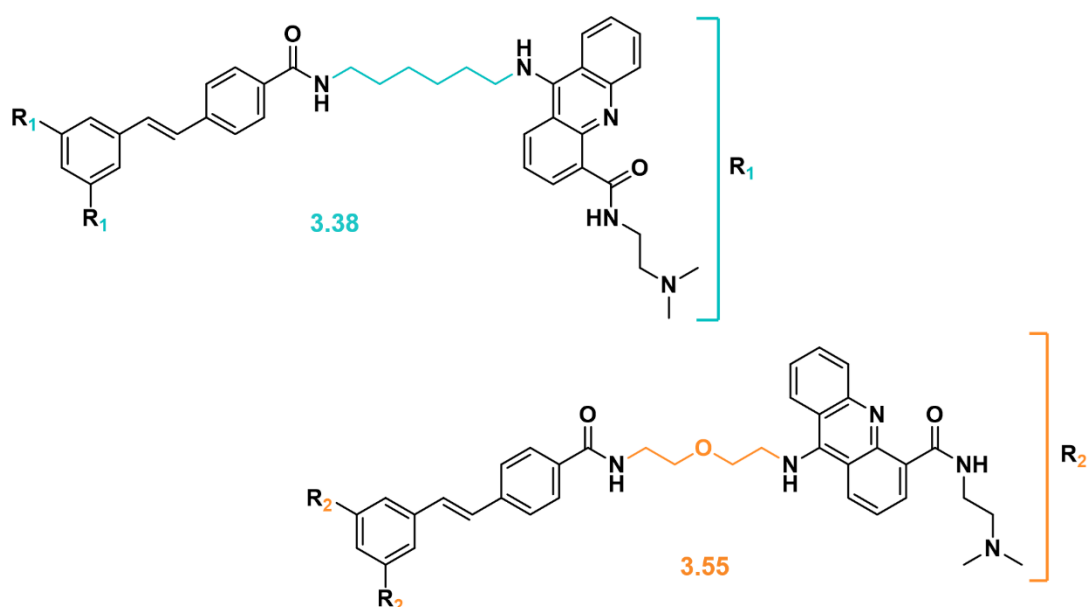
Scheme 3.13: Coupling reaction of pentafluorophenol trimer with acridine compound **3.57**

Coupling of the acridine groups onto the central unit was carried out in the same procedure followed for compound **3.38**. Compounds **3.43** and **3.57** were stirred overnight at room temperature in the presence of base. The product was purified via column chromatography and converted to the hexahydrochloride salt. This compound

was analysed by ^1H NMR, mass spectrometry, IR spectroscopy, melting point and 2D NMR experiments.

The ^1H NMR spectrum of this compound was similar to that of compound **3.38**, with some differences in the aliphatic region due to change in linkers. A total of forty two protons were calculated in the aromatic region, the order of signals remains the same as those described for compound **3.38**, with minor shifts. The high-resolution mass spectrum showed a mass of 1648.8396, matching the $[\text{M}+\text{H}]^+$ ion of the compound, 1648.8220.

The two final trimeric acridine structures designed and synthesised are shown in **Figure 3.14**, the next step involved running a series of biological assays to assess their DNA binding properties.



Scheme 3.14: Final structures of the two synthesised trimers

3.3.4 Evaluation of self-aggregation

Due to the large conjugated systems within the trimers, there was concern whether the compounds could form self-aggregates through π - π stacking. Self-assembly due to spontaneous aggregation of the molecules would lead to non-covalently bound stable ensembles, interfering with *in vitro* experiments. To investigate this, a UV spectroscopy titration was performed in water, from 10 to 100 μM of compound. In the case of no aggregation, when plotting the absorbance recorded at a specific wavelength against the compound concentration, a linear increase should be observed, due to a steady and consistent increase.

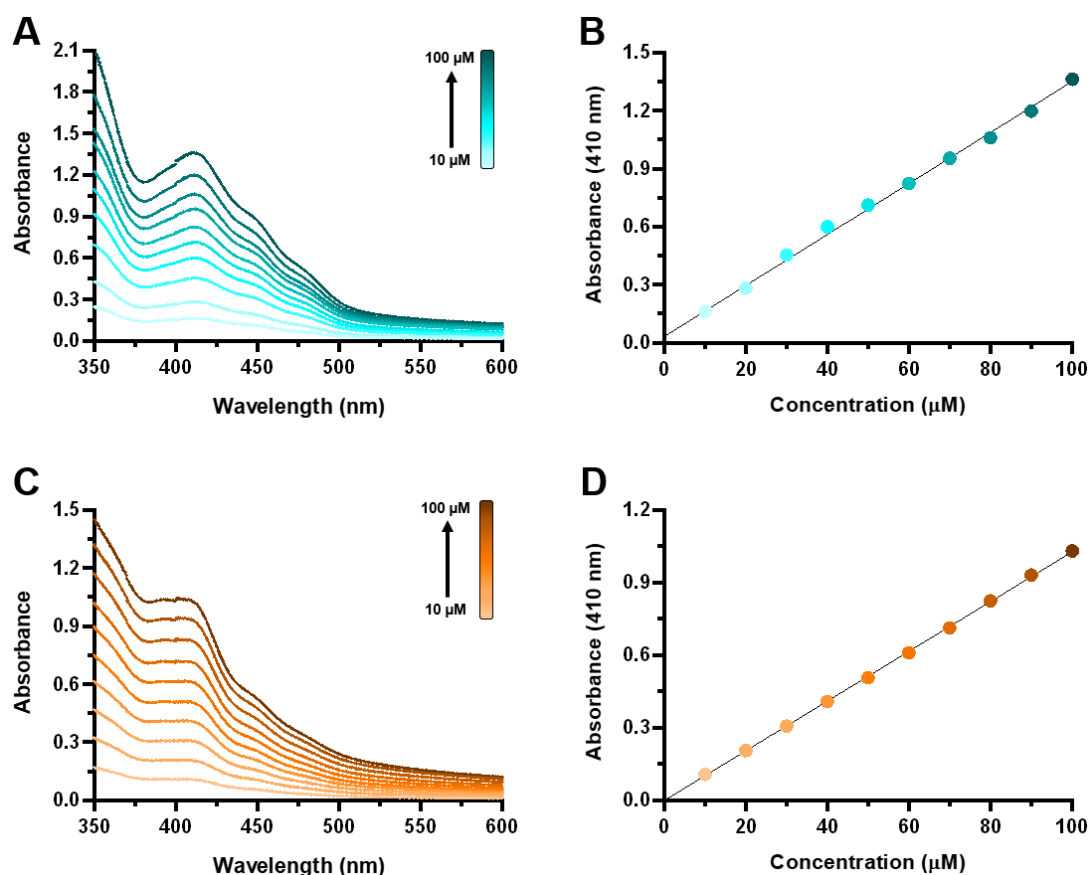


Figure 3.15: **A.** UV titration of the acridine trimer **3.38**, 1 μL each titration increasing the concentration by 10 μM , **B.** Absorbance recorded at 410 nm plotted against the compound concentration. **C.** Titration of compound **3.55**, each addition of 1 μL of compound led to a 10 μM increase. **D.** Absorbance recorded at 410 nm plotted against the concentration.

Results shown in **Figure 3.15** present linear graphs for both trimers, implying no self-aggregation was occurring at high concentrations of these compounds.

3.3.5 Investigation of duplex-to-duplex DNA binding

The next assay entailed confirmation of whether the compounds were able to bind with more than one acridine group simultaneously. The design relies on the acridines intercalating into each arm extension, maintaining their ability to intercalate different dsDNA molecules. In this case the duplex DNA sections of a three-way junction.

Similarly to the flexibly-linked acridine dimers, it was expected that these acridine trimers would be able to crosslink duplex DNA strands and so would be active in the gold nanoparticle assay described in **section 2.3.12**. As before, the gold nanoparticles were functionalised with thiolated dsDNA using a pH assisted procedure, followed by titration of a compound, scheme shown in **Figure 3.16**.

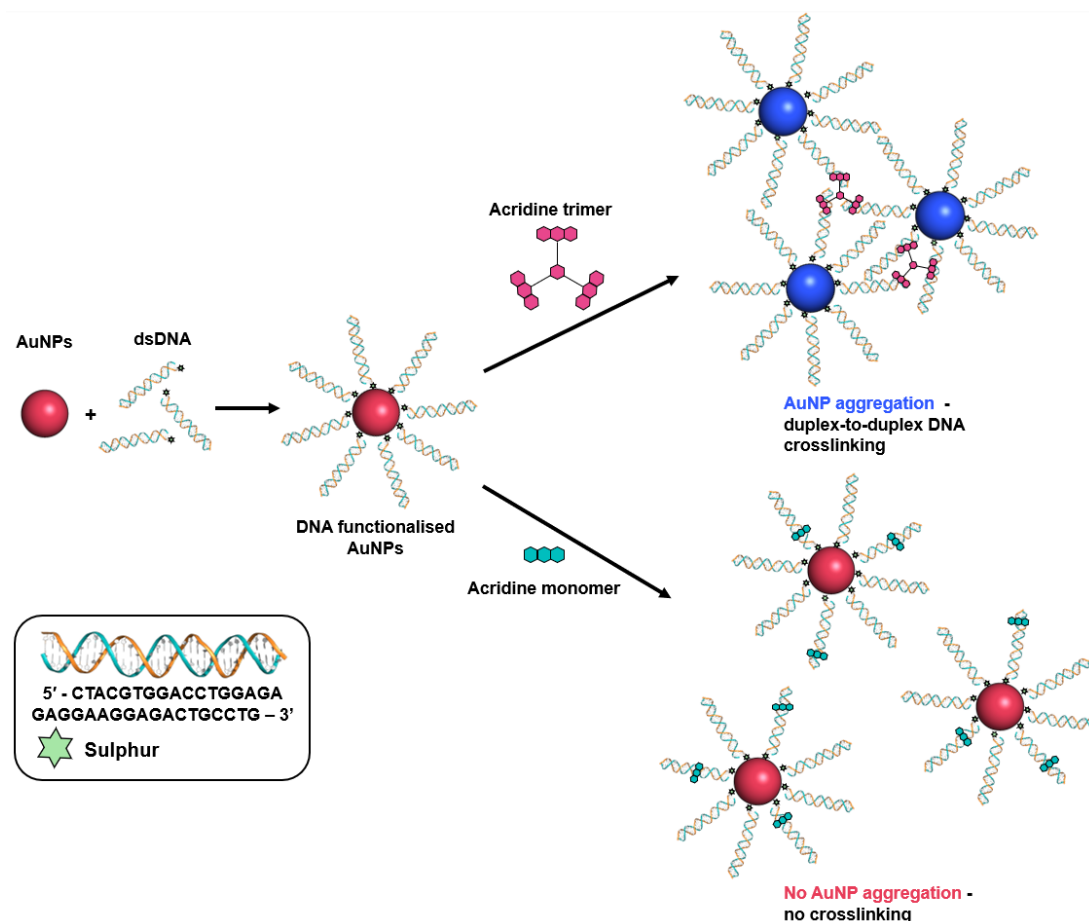


Figure 3.16: A diagram depicting a summary of a gold-nanoparticle based assay used to assess the compounds' abilities to carry out duplex-to-duplex DNA crosslinking.

A UV-Vis extinction spectrum of the dsDNA functionalised gold-nanoparticles was recorded after the addition of increasing amounts of a trimer **3.38**. Upon each addition, the sample was mixed for one minute, before recording the spectrum. The three signs suggesting aggregation were observed. Looking at the recorded spectrum (**Figure 3.17**), a red shift of the surface plasmon absorption band maxima peak is visible, as well as a decrease in intensity. Prior to any trimer addition, the dsDNA functionalised AuNP solution is a distinct red colour, however upon incubation with a trimer the colour rapidly disappears. These changes are expected if the compound is bringing the dsDNA-AuNPs together, forming clusters, therefore increasing in size, explaining the red shift of the peak, and aggregation, causing a decrease in intensity and a change in the colour of the solution.

To ensure the absorbance of the **3.38** had no effect on the assay results, a titration of **3.38** into PBS buffer was carried out. As seen in **Figure 3.17**, the compound's absorption wavelengths do not interfere with the calculated absorption relating to the gold-nanoparticles, due to no overlap in the wavelength regions of interest.

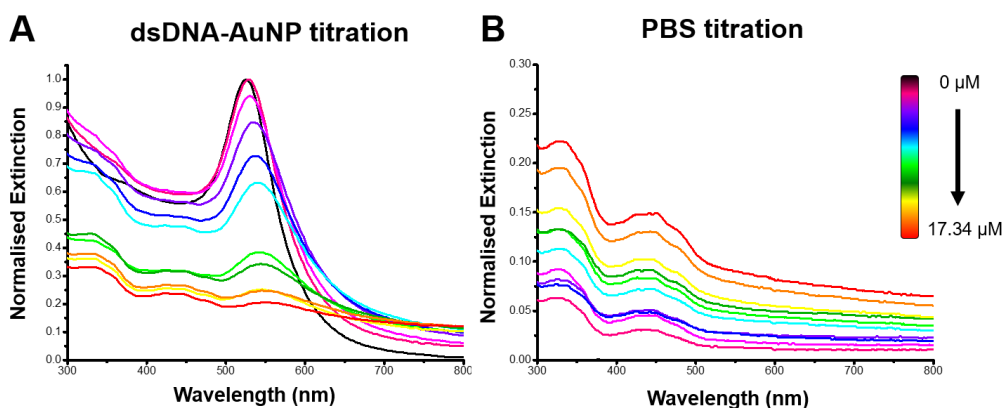


Figure 3.17: UV-Vis extinction spectrum recording of the titration of **3.38** **A.** to a solution of dsDNA functionalised gold nanoparticles (in PBS 10 mM, pH 7.4) and **B.** to PBS as a control.

In **Figure 3.17B**, the peak observed between 400 – 500 nm corresponds to the acridines, whereas the peak located between 300 – 400 nm correlates to the central tristyryl unit. Therefore, the changes observed in **Figure 3.17A** are confirmation that compound **3.38** can carry out duplex-to-duplex DNA crosslinking, necessary for our binding design. The same results were gathered for trimer **3.55**.

3.3.6 Assessment of three-way junction binding by PAGE assay

To assess whether the two trimers can bind to three-way junctions, a PAGE assay was performed. This technique separates molecules such as DNA, RNA and proteins, based on charge and size. Smaller and less charged molecules are expected to move further down the gel. Once again, polyacrylamide gels were chosen as they allow DNA complexes, such as three/four-way junctions, to maintain their structure during the assay. Three partially complementary oligonucleotides were chosen from literature⁴¹⁸, which assembled into a 3WJ with seven base pair long junction arms.

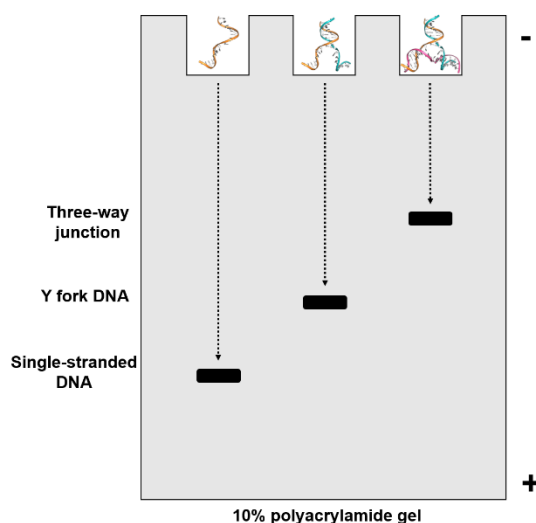


Figure 3.18: Representation of a gel electrophoresis experiment, where heavier DNA structures move slower down the gel, providing a qualitative way to identify the DNA shapes present in each well.

The three strands can exist in three states, as single-stranded DNA, as a Y fork, or as a three-way junction. These structures should appear at different parts of the gel due to their size and charge differences, with the smallest, ssDNA, moving the furthest, next the Y fork and finally the three-way junction is expected to move the least (**Figure 3.18**). Prior analysis suggests the three oligonucleotides chosen are unable to form a three-way junction at room temperature in the absence of a ligand, confirmed by our DNA only controls.

Three controls were included in the three-way junction gels. One well contained only the three oligonucleotides (C1), proving they are unable to form a three-way junction at room temperature in the absence of a ligand. The second control well held one of the oligonucleotides (C2), indicating where in the gel ssDNA would move to. Lastly, the last control involved the three oligonucleotides incubated with a known three-way junction binder, the Ni cylinder, which was kindly provided by Prof Hannon, to indicate the location of a formed three-way junction on the gel (C3).

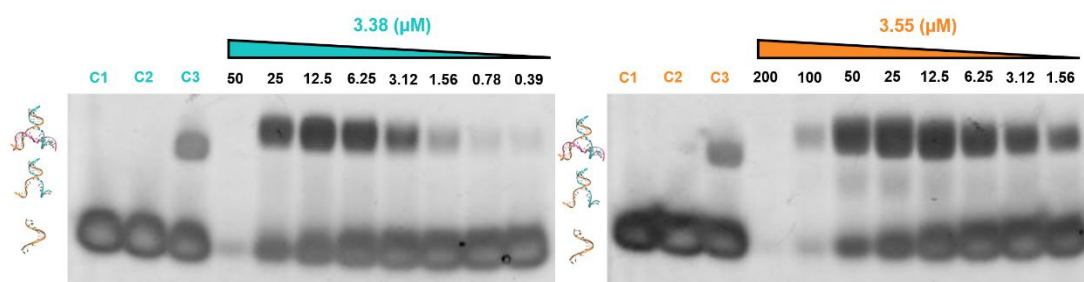


Figure 3.19: PAGE gel showing the assembly of a three-way junction upon increasing amounts of **3.38** and **3.55** in 1 μ M of three partially complimentary oligonucleotides, the solutions were allowed to incubate for 1 hour before the gel was run for 1 hour 45 minutes, at 50 V, at room temperature. **Controls - lane 1:** the three oligonucleotides, **lane 2:** one of the oligonucleotides indicating where ssDNA runs to on the gel, **lane 3:** a Ni helicate provided by Prof Michael Hannon, a known three-way junction binding compound, indicating the location of a 3WJ on the gel.

The gels were run at 1 μ M for each DNA strand and increasing concentrations of a compound. The compounds were left to incubate with the oligonucleotides for one hour at room temperature, before being transferred to the wells. Compound **3.38** was run in 1 in 2 dilutions from 50 μ M to 0.39 μ M, where all concentrations were able to form the three-way junction in a concentration-dependent manner (**Figure 3.19**). Precipitation of the compound was observed when testing higher concentrations of this compound. Additionally, compound **3.55** was also able to bind and stabilise three-way junctions. The concentrations chosen for this compound started at 200 μ M, carrying on with 1 in 2 dilutions, down to 1.56 μ M, a band corresponding to the three-way junction was observed in all combinations (**Figure 3.19**). The complete disappearance of bands at high concentrations of the two compounds is due to precipitation of the compound shortly after preparing the solutions.

To assess the trimers' selectivity for three-way junctions, the gel was repeated with four-way junction forming oligonucleotides. Four partially complimentary oligonucleotides were annealed in the presence of increasing trimer concentrations overnight and loaded onto a gel the next day. The final concentration of the DNA strands was 1.25 μM and for the compounds **1** in 2 dilutions from 200 μM to 1.56 μM . The gel was run for 2 hours at 50 V at room temperature.

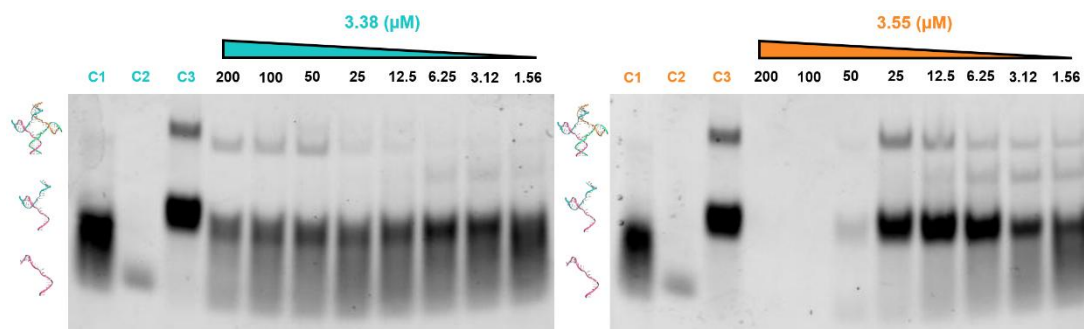
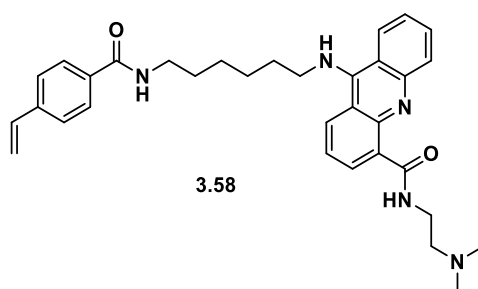


Figure 3.20: PAGE gel of four-way junction annealing in the presence of **3.38** and **3.55**. Concentrations tested 200, 100, 50, 25, 12.5, 6.25, 3.12 and 1.56 μM , at 1.25 μM DNA. **Controls** - **lane 1**: the four oligonucleotides, **lane 2**: only one of the oligonucleotides, **lane 3**: dimer **2.18** known to form a 4WJ from previous chapter.

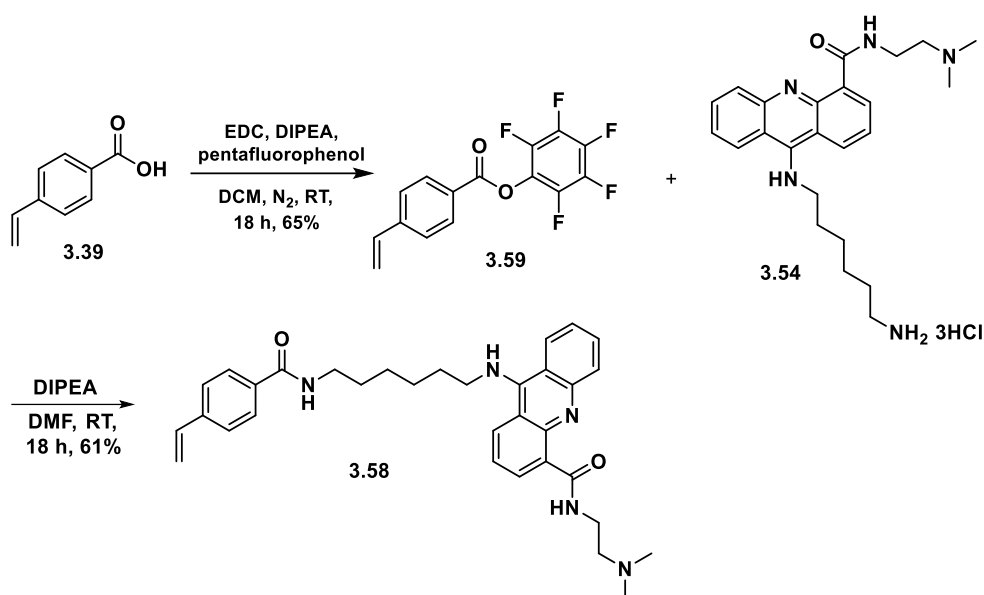
The gels obtained (**Figure 3.20**), suggest poor stabilisation of the four-way junction by the trimers, keeping with our hypothesis of their three-way junction preference.

Following the successful results of the trimers binding to the three-way junction, to confirm the trimeric structure was necessary for binding, a monomeric version was prepared.



Scheme 3.15: Monomer structure of trimer **3.58** to be used as a control

This compound was synthesised similarly to the trimers. A pentafluorophenol group was added onto 4-vinylbenzoic acid, which in turn was reacted with compound **3.54**, the acridine with a free amine at the end of the linker, to yield the monomer. The detailed scheme is outline below.



Scheme 3.16: Synthetic pathway to yield the desired monomer (3.58)

4-vinylbenzoic acid (**3.39**) was stirred with pentafluorophenol in dichloromethane in the presence of coupling reagent EDC and DIPEA as a base. The solution was stirred overnight at room temperature under nitrogen. Upon completion, the product was purified via column chromatography with DCM as the eluent. A white solid was isolated which was confirmed to be the desired product via NMR spectroscopy.

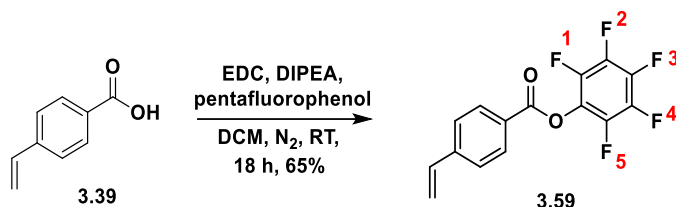
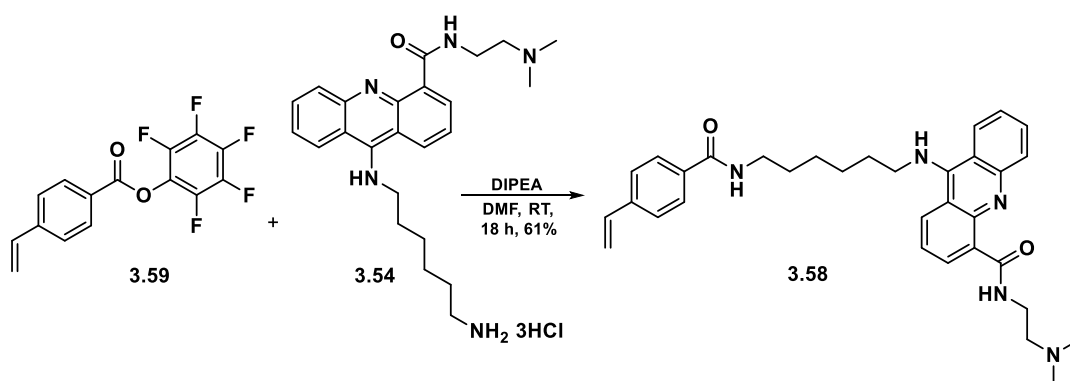


Figure 3.21: Addition of the pentafluorophenol group

The compound was analysed by ^1H NMR and ^{19}F NMR. The proton NMR spectrum consisted of five signals, very similar to that of the starting material, therefore a ^{19}F NMR was run to confirm the addition of the pentafluorophenol group. Three signals are observed, matching the three fluorine environments present. Due to the symmetry of the two *ortho*-coupled fluorines, F_1 and F_5 , correspond to the same signal, a multiplet integrating to two at -152.47 ppm, located the most downfield due to their proximity to the oxygen atom. A triplet at -158.04 ppm integrating to one fluorine is assigned to the para-coupled fluorine, F_3 , coupled to the meta-coupled fluorines. Lastly, a multiplet at -162.38 ppm integrating to two fluorines corresponds to the two meta-coupled fluorines, F_2 and F_4 .

Next, compound **3.59** was coupled with **3.54**, under the same conditions described in **scheme 3.9**.



Scheme 3.17: Coupling of compound **3.59** with acridine **3.54**

The two compounds were stirred overnight in DMF in the presence of DIPEA. The ^1H NMR of **3.58** confirmed the coupling, the signals matching the analysis of compound **3.38**. The difference in the two spectra concerns the alkene protons, which remain at positions 6.75 ppm, 5.86 ppm and 5.38 ppm, as in the spectrum of compound **3.36**. The low-resolution mass spectrum recorded a signal at 538.4, matching the $[\text{M}+\text{H}]^+$ ion at 538.3.

Once the synthesis of **3.58** was complete, a gel experiment was run assessing the monomer's three-way junction forming abilities. The experiment was carried out under the same conditions as **3.38**, the oligonucleotides were incubated with increasing amounts of compound for an hour. The gel was run at 50 V for 1 hour 45 minutes, stained with Sybr-gold stain for 30 minutes and then imaged.

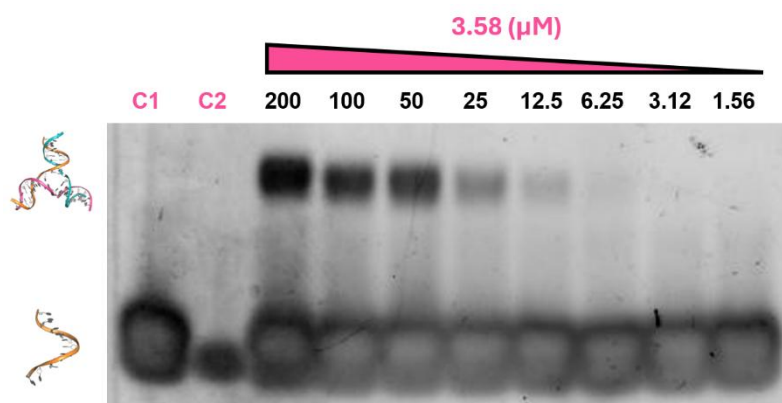


Figure 3.22: PAGE gel of the acridine monomer **3.58**, at 1 in 2 concentrations from 200 to 1.56 μM , added to 1 μM three oligonucleotides. **Controls - lane 1:** three oligonucleotides unable to form a stable 3WJ in the absence of a ligand, **lane 2:** only one of the oligonucleotides highlighting the locations of the ssDNA strands in this experiment.

Upon visualisation of the gel (**Figure 3.22**), at high concentrations of the monomer a 3WJ band is visible, however, a large amount of ssDNA can also be observed. The monomer is able to induce assembly of the 3WJ down to 12.5 μM whilst a 3WJ band is visible down to 0.39 μM in the presence of trimer **3.38** (**Figure 3.19**).

The experiment was also repeated with the dimeric version of the compound **2.18**, whose synthesis was described in **section 2.3.1**. The conditions were maintained, with 1 in 2 dilutions of the compound, from 200 μM to 1.56 μM , with the final DNA concentration at 1 μM .

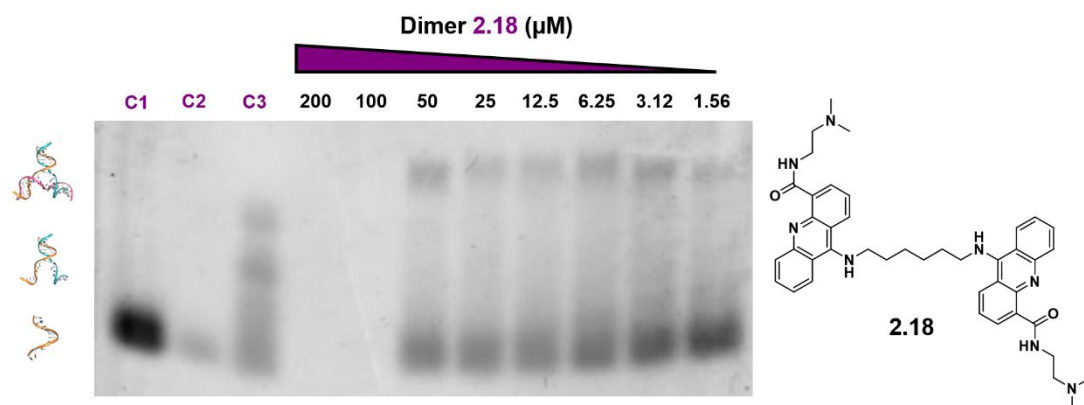


Figure 3.23: PAGE gel of the acridine dimer, at 1 in 2 concentrations from 200 to 1.56 μM , added to 1 μM three oligonucleotides. **Controls - lane 1:** three oligonucleotides unable to form a stable 3WJ in the absence of a ligand, **lane 2:** only one of the oligonucleotides highlighting the locations of the ssDNA strands in this experiment, **lane 3:** Ni cylinder stabilising three-way junction.

The gel (**Figure 3.23**) indicates some 3WJ stabilisation from the dimer, however the low intensity of the bands suggests non-specific binding as well.³⁸⁵ Due to the qualitative nature of the assay a comparison between three-way junction formed in the presence of the monomer, the dimer or the trimer is not possible when comparing different gels. Therefore, an experiment was run to simultaneously compare the monomer, dimer and trimer's abilities to form three-way junctions. The compounds bearing the six-carbon linker were chosen for consistency, as seen in **Figure 3.24**.

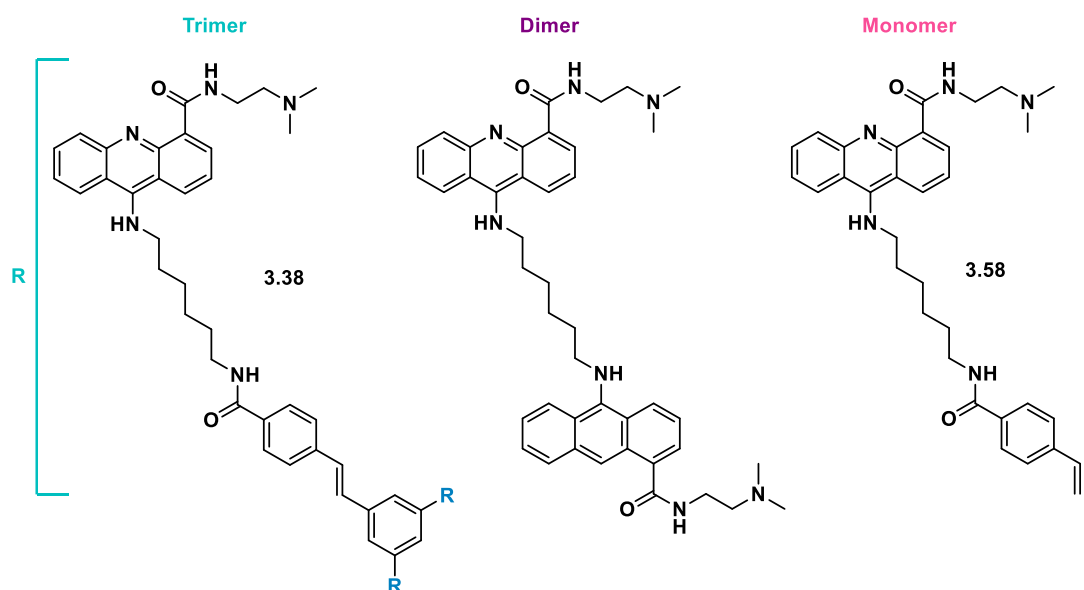


Figure 3.24: Structures of three acridine compounds, bearing three (**3.38**), two (**2.18**) or one (**3.58**) acridine moieties, all bearing a six-carbon chain as a linker for similarity

The concentrations chosen for all three compounds were 50, 25 and 12.5 μM , the compounds were allowed to incubate with 1 μM three-way junction for one hour before adding them to the wells. The gel was run for 1 hour 45 minutes, at 50 V in TBE buffer.

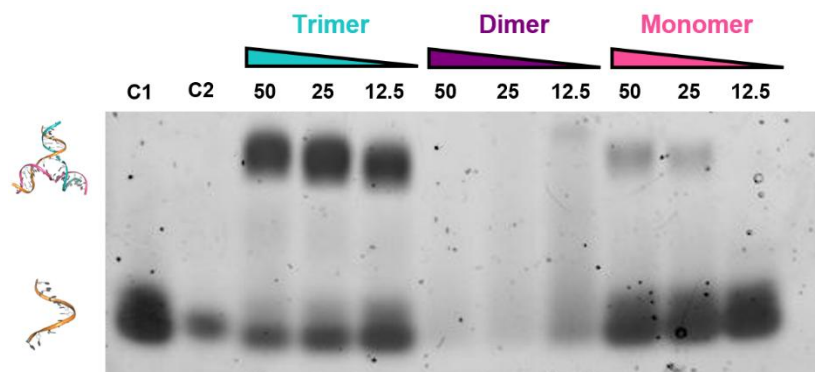


Figure 3.25: PAGE gel of **3.38**, **2.18** and **3.58**, at 50, 25 and 12.5 μM , added to 1 μM of three oligonucleotides, showing a considerable increase of three-way junction only in the presence of the trimeric structure. **Controls - lane 1:** three partially complementary oligonucleotides unable to form 3WJ, **lane 2:** only one oligonucleotide, indicating where the ssDNA runs to on the gel.

Two control wells were included, one contained the three oligonucleotides (C1) to confirm they are unable to form a three-way junction in the absence of any compound. In the second control well only one of the oligonucleotides was added, to mark the location the single-stranded DNA molecules should appear at. The gel was left to stir for 30 minutes in Sybr-gold DNA stain, followed by imaging to observe the bands, with the resulting gel shown in **Figure 3.25**. At the selected concentrations, the monomer forms a small amount of three-way junction. The dimer seemed to aggregate and precipitate at these concentrations, therefore the bands seen are very faint. The trimer however is seen to form mainly three-way junction, with a small amount of ssDNA remaining. These results confirm that the trimer is considerably more selective towards three-way junction compared to its dimeric and monomeric versions.

When comparing the results gathered in this section to literature, **3.18** and **3.55** induce 3WJ formation and stabilisation at lower concentrations than reported small molecules to date. The best compound of the azacryptands, introduced in **section 3.1.6**, assessed through an EMSA assay is TrisBP, inducing 3WJ formation as low as 2.5 μM .⁴⁰⁰ In comparison, **3.18** and **3.55** can go down to at least 1.56 μM , with potentially **3.55** reaching lower concentrations, **Figure 3.19**. The cylinders discussed in **section 3.1.2** perform similarly, with concentrations around 1 μM . However, lower concentrations are required by peptide helicates, with the arginine helicates, presented in **section 3.1.3**, carrying out 3WJ assembly at a 50 nM concentration.

3.3.7 Investigation of DNA binding stability via a FRET-based melting assay

Upon confirmation that the trimeric compounds can promote 3WJ formation, the next step involved investigating their ability to stabilise folded DNA junctions against thermal denaturation. The FRET-melting assay described in **section 2.3.15**, could provide insight into the stabilising properties of the compounds in relation to different DNA structures. The assay was repeated with the same concentration of double-stranded DNA, a three-way junction as well as a four-way junction. As described previously, the doubly labelled folded DNA molecules were heated from 25 °C to 95 °C, one degree per minute, in the absence and presence of ligands.

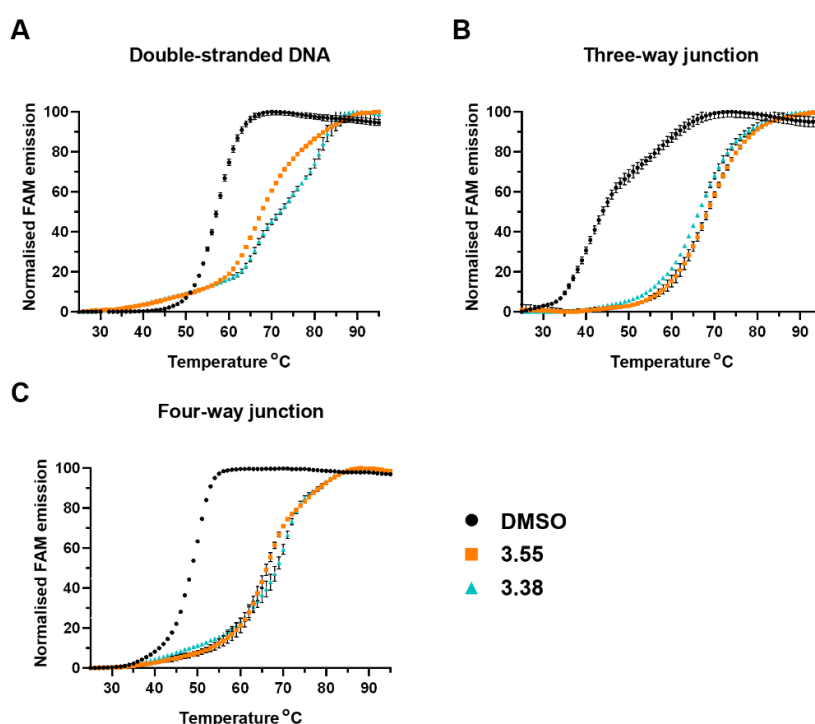


Figure 3.26: FRET-melting curves of experiments performed from 25 to 95 °C with 200 nM of **A.** double-stranded DNA, **B.** a three-way junction, **C.** a four-way junctions, in the presence of DMSO (black, control), 10 μ M **3.38** (teal) and 10 μ M **3.55** (orange) ($n=3$).

As the DNA structures are doubly labelled with the FRET pair FAM and TAMRA, the melting of the DNA is measured by monitoring of the FAM signal, directly associated with the unfolding of the structures (**Figure 3.26**). The data is summarised in **Table 4**, stating the temperature calculated as the midtransition point of denaturation, as well as the difference in that temperature upon the binding of a ligand, however certain melting curves indicate two transition events, where two melting temperatures are reported. Compound **3.38** steadily increased the stability of the dsDNA and four-way junction by approximately the same amount at 10 μ M, 14 - 16 °C, doubling the increase regarding the three-way junction, specifically to 29 °C. Interestingly,

compound **3.55** mildly stabilised the dsDNA and four-way junction by 8 °C and 15 °C accordingly, with a double transition reported during the 4WJ melting, potentially due to stabilisation of an intermediate formed during the melting by the compound (ΔT_{m2} = 29 °C). In contrast, the ΔT_m calculated for the three-way junction in the presence of compound **3.55** was 32 °C, a significant increase in the temperature required to denature the junction. The melting curve of the 3WJ in the absence of a ligand suggests two events are taking place during the unfolding of the DNA structure corresponding to two T_m values. However, upon addition of the compounds the melting occurs with one transition, suggesting that the compound shifted the structure's conformation equilibrium, into a more stable structure, resulting in one melting event. These results showed great promise regarding potential selectivity of these compounds for three-way junction over the other DNA forms.

Table 4: Summary of FRET-melting results if experiments carried out with FAM and TAMRA labelled double-stranded DNA, three-way junction DNA and four-way junction DNA, in the presence of DMSO, 10 μ M **3.38** trimer and 10 μ M **3.55** trimer ($\pm$ SEM) ($n=3$).

Ligand	dsDNA			Three-way junction			Four-way junction		
	T_{m1}	ΔT_{m1}	ΔT_{m2}	T_{m1}	ΔT_{m1}	T_{m2}	T_{m1}	ΔT_{m1}	ΔT_{m2}
DMSO	57 $\pm$ 1	-	-	40 $\pm$ 2	-	60 $\pm$ 1	51 $\pm$ 2	-	-
3.38	72 $\pm$ 2	14 $\pm$ 2	9 $\pm$ 1	69 $\pm$ 2	29 $\pm$ 2	-	66 $\pm$ 2	16 $\pm$ 2	-
3.55	66 $\pm$ 1	8 $\pm$ 1	-	72 $\pm$ 3	32 $\pm$ 3	-	65 $\pm$ 2	15 $\pm$ 1	29 $\pm$ 2

The melting temperatures recorded for **3.18** and **3.55** for 3WJs, 29 and 32 °C respectively, are significantly higher than ΔT_m values reported for other 3WJ stabilisation compounds to date. The highest temperature reported corresponds to stabilisation by 4,4'-TrisBP, **Figure 3.9**, with a ΔT_m value of 19 °C⁴⁴⁵, followed by NQO1-capped helicate, discussed in **section 3.1.2** with a ΔT_m value of 18 °C.⁴²³

3.3.8 Investigation of three-way junction selectivity with competition melting assay

As these results were carried out in a scenario where only one DNA form was present, a competitive DNA structure was added to the assay, to investigate whether binding was selective. When the ligand is bound to the 3WJ, its stabilising effects cause an increase in the melting temperature. Therefore, if an unlabelled second DNA structure was added, and the ligand showed a preference for the latter, the junction's melting temperature would decrease, due to the loss of ligand binding, and as a result, loss of ligand stabilisation.

Firstly, it was important to assess the trimers' selectivity for 3WJs over duplex DNA. The duplex competitor chosen was self-complementary and made up of 26 base pairs whose melting temperature is $T_m = 70.5\text{ }^{\circ}\text{C}$. As a result of the duplex DNA's high thermal stability, it should remain folded at temperatures at which unbound 3WJ will unfold. No tags were added as to not interfere with the FRET signals related to the 3WJ. The assay was run with 1, 10 and 50 equivalents of the dsDNA competitor in relation to the 3WJ, to account for differences in DNA length and potential binding locations. The data gathered is presented in **Figure 3.27A**, highlighting the effect of increasing amount of dsDNA to the 3WJ's T_m values calculated. The compound is added at $10\text{ }\mu\text{M}$, a 50-fold excess to the labelled DNA, and even upon addition of the same 50-fold excess of dsDNA, the 3WJ is still stabilised higher than $10\text{ }^{\circ}\text{C}$, indicating good selectivity of the trimers to 3WJ.

An unlabelled four-way junction was also tested as a competitor at 10 equivalents (**Figure 3.27B**). Finally, the 3WJ selectivity of the trimers was also tested in the reverse melting competition assay, where 1 and 10 equivalents of unlabelled 3WJ were added as a competitor to FAM/TAMRA labelled dsDNA. A large decrease is observed, with the calculated ΔT_m values reaching below $5\text{ }^{\circ}\text{C}$, suggesting a preference for 3WJ.

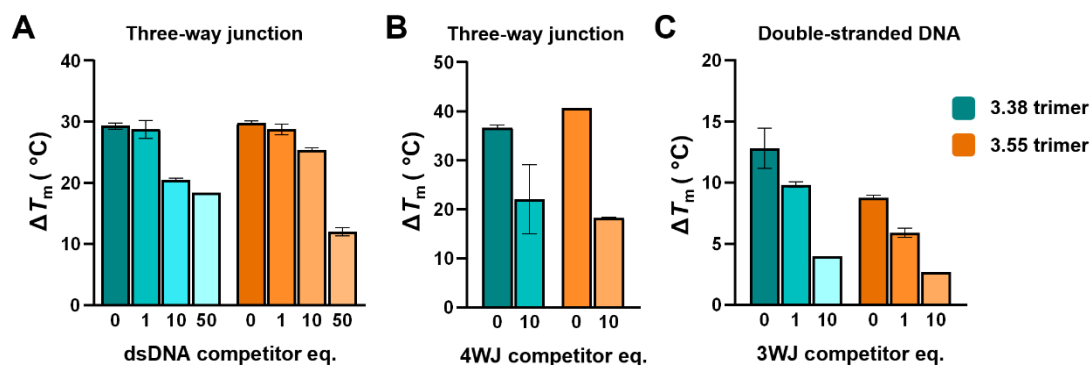


Figure 3.27: Recorded results from the competitive FRET-melting experiments with **A.** FAM/TAMRA labelled 3WJ (200nM) in the presence of $10\text{ }\mu\text{M}$ **3.38** and **3.55** trimers and increasing concentrations of duplex ds26 competitors, 1, 10 and 50 equivalence, **B.** FAM/TAMRA labelled 3WJ (200nM) in the presence of $10\text{ }\mu\text{M}$ **3.38** and **3.55** trimers and 10 equivalence of unlabelled 4WJ, and **C.** FAM/TAMRA labelled dsDNA (200nM) in the presence of $10\text{ }\mu\text{M}$ **3.38** and **3.55** trimers and increasing amounts of unlabelled 3WJ, 1 and 10 equivalence ($n=3$) ($\pm\text{SEM}$).

Analysing the results from **Figure 3.27**, the two trimers, display high selectivity for 3WJ DNA over dsDNA, as the ΔT_m only largely decreases when 50 equivalents of competitor dsDNA is added. Even in the latter case the 3WJ is still stabilised around $15\text{ }^{\circ}\text{C}$, a significant amount. Regarding the addition of competitor 4WJ, the ΔT_m values drop to half, however still measuring a high increase of around $20\text{ }^{\circ}\text{C}$. When testing the compounds selectivity when bound to dsDNA with the addition of unlabelled 3WJ,

a considerable decrease is measured, the compounds seem to prefer 3WJ binding as the denaturing temperature of the dsDNA dropped to under 5 °C.

3.3.9 Measurement of DNA binding constants by fluorescence titration

A fluorescence titration was carried out with both trimers, to calculate binding constants with duplex, 3WJ and 4WJ DNA. The procedure described in **section 2.3.16** was performed, keeping the compound concentration at 1 μM , with the DNA final concentrations ranging from 0.1 – 5 μM . K_D values were measured through Graphpad Prism, presented in **Figure 3.28**.

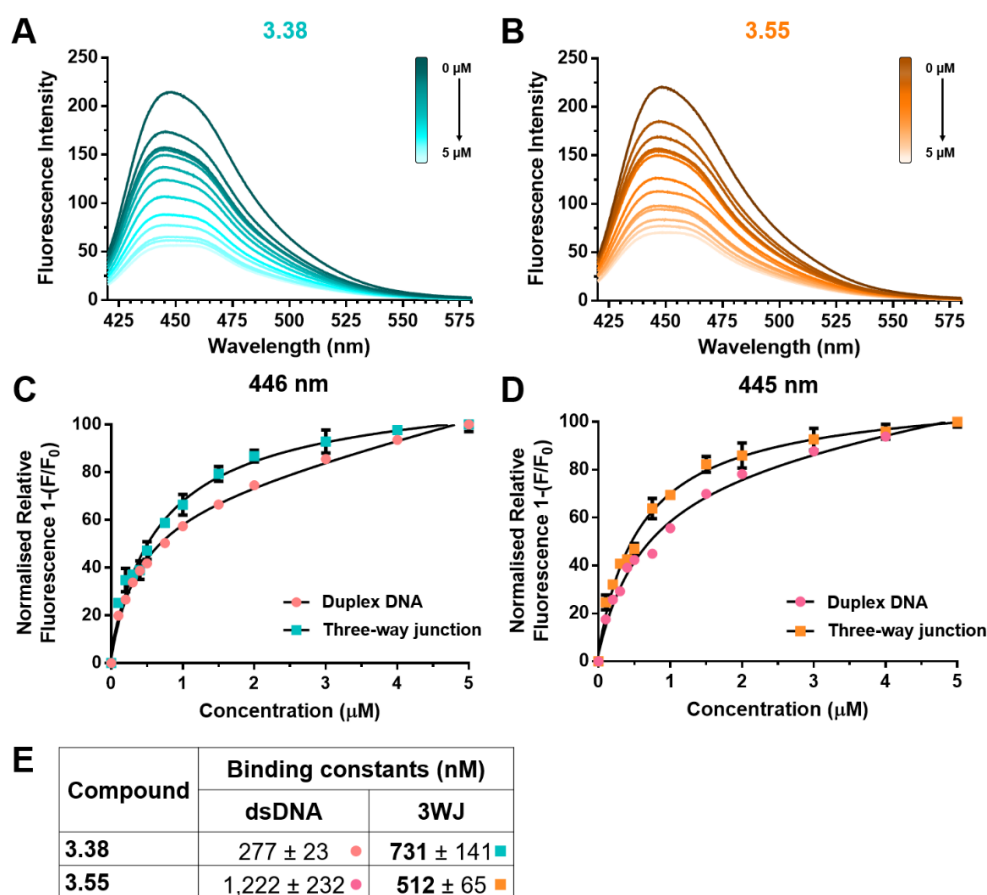


Figure 3.28: Spectral scans of DNA titrations of 0.1 μM , 0.2 μM , 0.3 μM , 0.4 μM , 0.5 μM , 0.75 μM , 1 μM , 1.5 μM , 2 μM , 3 μM , 4 μM 5 μM to A. to 1 μM **3.38**, B. to 1 μM **3.55**, C. dose-response curve plotted of the fluorescence intensity recorded for each experiment at 446 nm for **3.38**, D. dose-response curve plotted of the fluorescence intensity recorded for each experiment at 445 nm for **3.55**, E. table of binding constants calculated through GraphPad Prism version 8.0 fitted to a one-site model, $Y=B_{\text{max}}*X/(K_d+X) + NS*X + \text{Background}$ ($n=3$) ($\pm\text{SEM}$).

Titration with the 3WJ structure are shown above, both compounds follow a similar quenching pattern, with K_D values of 0.51 and 0.73 μM , for compound **3.38** and **3.55** respectively. This shows strong binding to the 3WJ structure by both compounds, accompanied by great stabilisation of the structure, as highlighted from the melting assay. Additionally, **3.38** exhibited good binding to the duplex DNA, considerably higher than **3.55**, with these results mirrored in the melting experiment.

3.4 Conclusions

This chapter presents a review of the 3WJ binding compounds published to date, inspiring the design of two novel tri-acridine intercalators, **3.38** and **3.55**. The compounds were designed with a 1,3,5-tristyrylbenzene centre, extending symmetrically into three arm extensions, bearing 9-aminoacridine-4-carboxamide groups. The synthetic pathway analysed is made up of a total of ten steps, accompanied by detailed characterisation of each product.

A gold-nanoparticle based assay was performed, following the synthesis of gold nanoparticles, further functionalised with dsDNA. The assay confirmed the trimers could bind simultaneous duplex DNA molecules through the acridine moieties, necessary for the intended mode of binding to the three-way junction.

Gel electrophoresis experiments demonstrated both trimers inducing the assembly of a three-way junction, when incubated with three partially complimentary oligonucleotides, at nanomolar range concentrations. The compounds were further assessed for any 4WJ binding, results supporting there was none. Finally, when compared to an acridine dimer and monomer, the trimer performed significantly better, highlighting the promise of the design.

The effect of the compounds on the stability of a 3WJ was measured through a FRET-based melting experiment. The increase in the melting temperature was recorded around 30 °C, demonstrating very strong stabilisation upon binding of the compounds, 10 °C more than the highest melting temperature increase reported in literature ($\Delta T_m = 19\text{ °C}$).⁴⁴⁵ A competition melting experiment indicated a small loss in stabilisation upon addition of an unlabelled dsDNA competitor, supporting strong selectivity for the 3WJ.

Chapter 4

Cellular evaluation of acridine dimers and trimers targeting four-way and three-way DNA junctions

4.1 Introduction

4.1.1 DNA damage

Maintaining genomic stability is a critical priority of cells to guarantee survival and reproduction. It ensures cell division results in the accurate duplication of the genome passed onto the daughter cells, containing the same genetic material as the parent cell. Alterations to the genetic code can disrupt cellular biological processes, preventing the cell from functioning properly, possibly inducing carcinogenesis or cell death.⁴⁵³

DNA damage repair is a critical factor in cancer development and progression, due to its role in maintaining genomic stability through DNA replication and transcription.^{454–456} Endogenous and exogenous stresses can generate various types of DNA damage in cells, including base pair alterations, errors in DNA replication or damage to double helix strands. Exogenous factors include environmental, physical and chemical agents that damage DNA, such as heat, UV, ionising radiation, DNA alkylating and crosslinking agents.^{457–467} Endogenous DNA damage can occur from the DNA participating in hydrolytic and oxidative reactions with water and reactive oxygen species (ROS).⁴⁶⁸

There are several types of DNA damage described as 1) base damage, 2) sugar damage, 3) DNA cross-linking, 4) clustered damaging sites, 5) single-strand breaks (SSBs) and 6) double-stranded breaks (DSBs).⁴⁶⁹ In most cases, base damage is caused by ROS generated due to oxidative stresses,^{470,471} whilst sugar damage is often triggered by free radicals, produced in cells with metabolic reactions and by exogenous agents.^{472,473} DNA protein cross-links are formed by chemical crosslinking agents such as cisplatin,^{467,474} UV,^{475,476} heavy metal ions⁴⁷⁷ and inhibitors of DNA-metabolising enzymes (topoisomerase).⁴⁷⁸ Clustered DNA damage sites contain two or more lesions formed within one to two helical DNA turns caused by the same damaging factor.⁴⁷⁹ They are most commonly the result of ionising radiation, causing multiple damage sites due to uneven depositing of energy along its track.^{480,481}

It has been reported that approximately 70,000 instances of DNA damage arise in a human per day, with most of the lesions being single-strand breaks.^{482,483} SSBs can occur directly through breakdown of the DNA backbone, or indirectly as intermediates or products of DNA repair and metabolic pathways.⁴⁸⁴ The most common cause of SSBs is deoxyribose oxidation, which, following hydrogen abstraction, can result in SSBs carrying a non-ligatable fragmented 3' termini.⁴⁸⁵ Oxidation of the DNA sugar

can also indirectly cause a SSB, after losing its nucleobase, resulting in cleavage of the remaining backbone by AP-endonuclease during the base excision repair (BER) pathway⁴⁸⁶. SSBs can also be induced indirectly by other DNA excision repair pathways, including nucleotide excision repair,⁴⁸⁷ ribonucleotide excision repair⁴⁸⁸ and mismatch repair.⁴⁸⁹ In these repair pathways, SSBs are formed as intermediates, and are subsequently resealed, however, an error in the pathway can lead to the repair being skipped, requiring detection and repair of the lesion by SSB repair pathways. Furthermore, SSBs are also formed as intermediates in topoisomerase mechanisms, causing breaks in the DNA during replication and transcription to remove torsional stress of the helix or topological constraints. Upon completion, topoisomerases religate the DNA breaks, however, this process may be skipped if the complex collides with DNA/RNA polymerases, or other DNA structures, resulting in an unrepaired SSB.^{490–492}

The most cytotoxic forms of lesion are double strand breaks (DSBs), in which the sugar-phosphate backbone of the two complementary DNA strands is broken simultaneously. DSBs are commonly formed due to exposure to ionising radiation, chemotherapeutic drugs, DNA replication stress and defects in transcription.⁴⁹³ Ionising radiation generates extensive base damage, and can also lead to SSBs by producing radiolysis radicals that attack the DNA backbone, high doses of irradiation can result in SSBs in complementary strands, leading to a DSB.^{494–497} Chemotherapeutic agents that can cause DSBs are DNA-alkylating agents, cross-linking agents, radiomimetic compounds and topoisomerase inhibitors.⁴⁹³ Moreover, SSBs are often intermediates in DNA metabolic processes, collision of the replication fork with these DNA sections can result in their conversion into DSBs, hindering the replication process.^{498–500} DSBs are one of the major factors of genomic instability, therefore the cells have specific DNA repair mechanisms in place to repair them.

4.1.2 Double strand break repair mechanisms

Double strand breaks are repaired by two types of pathways, homologous recombination (HR) and non-homologous end joining (NHEJ).⁵⁰¹ HR is a more conservative and error-limiting mechanism, repairing the DNA through recombination between sister chromatids. This repair mechanism is largely restricted to the S and G2 phase of the cell cycle, as that is when sister chromatids are available.^{502,503} On the other hand, NHEJ can occur throughout the cell cycle, modification and ligation of the broken DNA ends is carried out with very little or no homology, without a template, often forming small insertions or deletions.

4.1.2.1 Homologous Recombination (HR)

Homologous recombination was first described by Meselson, his work showing that recombination between genomes of bacterial viruses was performed with the breaking and rejoining of parental DNA.^{504,505} However, the mechanism was not understood until Holliday proposed his model in 1964.²²⁹ Extensive research led to the uncovering of three distinct HR processes, called double-strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR), outlined in **Figure 4.1**.⁵⁰¹ Upon detection of a DSB, a single-strand section is cut from both the DSB's ends, producing terminal 3'-OH single-stranded DNA tails. The resected DSB ends pair with the homologous template, forming a D-loop structure, to prime the synthesis of the repaired DNA. In somatic cells, use of the sister chromosome is preferred as the repair template, over the homologous chromosome. Upon formation of the D-loop structure, the remaining steps of the pathway differ in each HR process. In the DSBR pathway, the D-loop structure is further stabilising by capturing the remaining 3' terminus, forming a double Holliday junction structure. In the SDSA pathway, no HJ structure is formed, instead HR proceeds through extension and release of the 3' single-stranded tail from the template, continuing with the DNA synthesis of the complementary strand. In the BIR pathway, DNA synthesis continues via the formation of a replication fork, repairing the strands in an asynchronous manner, as a leading and lagging strand.

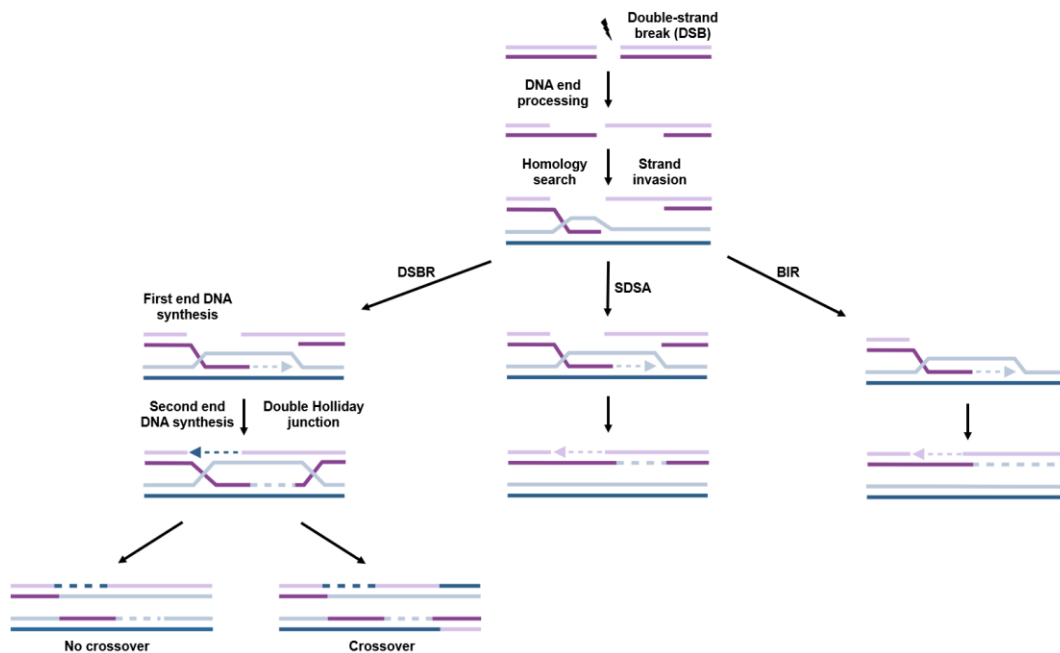


Figure 4.1: Representation of the homologous recombination pathway in response to a double strand DNA break. The repair is carried out by one of three DNA processes, double strand break repair (DSBR), synthesis-dependent strand annealing (SDSA) or via break-induced replication (BIR)

One of the protagonists of HR in humans is protein Rad51, involved in the search and strand exchange of homologous sequences.⁵⁰⁶ Once the 3' ssDNA tails have been generated, Rad51 is loaded onto the DNA, by mediators including BRCA1-BRCA2-PALB2, forming a Rad51 nuclear fiber structure, the Rad51-ssDNA filament.⁵⁰⁷ Rad51 then promotes the formation of the D-loop structure by directing the ssDNA to the homologous DNA template. Upon association of the ssDNA with the template, Rad51 breaks away from the DNA strand, freeing its 3' terminal sequence, subsequently used as the primer for DNA synthesis.⁵⁰⁸

Another important regulator of DNA repair via HR, is kinase ATM (ataxia telangiectasia mutated), in relation to the MRN complex, and 53BP1. The MRN complex, made up of MRE11, RAD50, NBS1, is continuously scanning the genome for damage sites, searching for free DNA ends.^{509–511} Upon detection of a DSB, MRN recruits ATM to the identified DNA damage site, ATM then phosphorylates several downstream repair proteins, including p53, PALB2, Rad51 and histone H2AX⁵¹². The phosphorylation of H2AX acts as an indicator of DSB identification, leading to the recruitment of chromatin remodelling, and other end restriction associated, factors. MRN also initiates end resection through the MRE11⁵¹³ nuclease and recruitment of CtIP,⁵¹⁴ forming the short tracts of ssDNA. CtIP in turn recruits DNA2 and EXO1, to carry out end processing and generate long stretches of ssDNA.^{515,516} Additionally, a recent study demonstrated that CtIP can bridge two DNA ends, likely the broken 3' ends together until other repair factors arrive.⁵¹⁷ ATM and cyclin-dependent kinase (CDK) phosphorylate CtIP, initiating MRE11 and CtIP nuclease activity whilst destabilising Ku, involved in the NHEJ pathway.^{518,519} The phosphorylation of these proteins promotes HR while inhibiting NHEJ.

ATM is further involved in HR through a secondary pathway, in relation to 53BP1, a regulator involved in the choice of HR or NHEJ for a DSB repair. 53BP1 favours NHEJ, preventing HR by inhibiting end resection by recruiting Shieldin via the RIF1-53BP1 complex, blocking the first step of the HR pathway.⁵²⁰ In G1 phase, 53BP1 is phosphorylated by ATM, leading to the promotion of NHEJ by RIF1-shieldin recruitment. However, in G2 phase, 53BP1 phosphorylation is significantly decreased, therefore HR is allowed to proceed.⁵²¹

4.1.2.2 Nonhomologous end joining (NHEJ)

Double strand breaks are also repaired via nonhomologous end joining, a process in which DNA ends are brought together by a multiprotein synaptic complex and directly ligated, with minimal reference to DNA sequence (**Figure 4.2**). Upon detection of a

DSB, a Ku heterodimer, made of subunits Ku70 and Ku80, binds to the exposed DNA ends.^{522,523} The dimer recruits the DNA-dependent protein kinase catalytic unit (DNA-PKcs) to the damage site.⁵²⁴ The DNA-PKcs recognises the DNA-bound Ku dimer, and they form a complex referred to as the DNA-PK complex or holoenzyme⁵²⁵. The DNA-PK complex promotes the formation of a synaptic complex, preparing the DNA ends for ligation with the recruitment of several end-processing factors, including the nuclease Artemis, and DNA polymerases λ and μ ⁵²⁶. Upon preparation of the DNA ends for ligation, DNA-PK recruits XRCC4, XLF and LIG4^{527–529}. The linkage of the disconnected DNA ends is catalysed by DNA ligase IV (LIG4), which forms a complex with XRCC4 and XLF, repairing the damaged DNA site⁵³⁰.

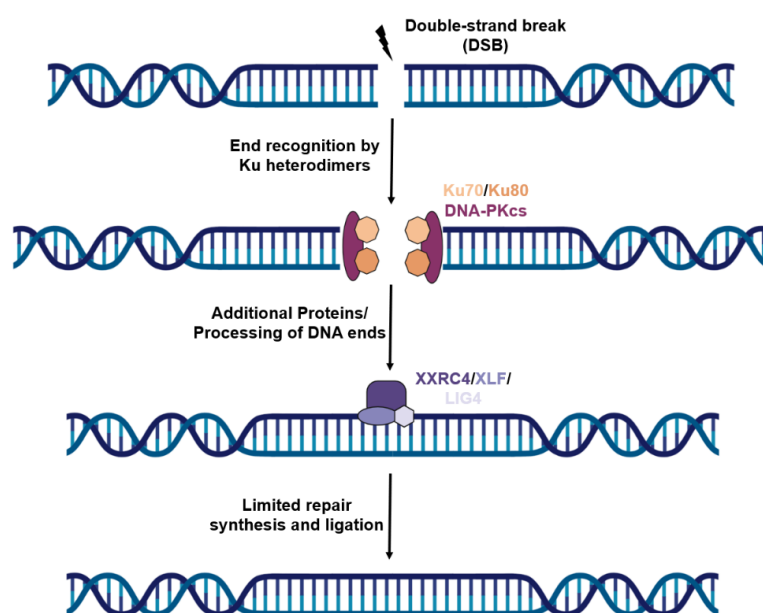


Figure 4.2: Repair of a double strand break via the nonhomologous end joining pathway

4.2 Chapter aims

This chapter aims to assess the biological relevance of the acridine dimers and trimers discussed in chapters 2 and 3. Firstly, the cytotoxicity of the compounds were measured using an enzymatic MTS assessment, exploring their effect on cell viability in two cancer cell lines. Additionally, the co-treatment of the compounds with known DNA repair inhibitors was explored, to determine whether they could induce greater cytotoxicity through synergy.

4.3 Results and Discussion

4.3.1 Cell viability assay – Acridine dimers

A colorimetric cell viability assay was performed to understand the effects of the acridine compounds on the proliferation of cancer cells. Dimethylthiazol-carboxymethoxyphenyl-sulfophenyl tetrazolium (MTS) is a yellow dye bearing a tetrazole group, that undergoes reduction by mitochondrial dehydrogenases upon cellular uptake, producing purple formazan crystals (**Figure 4.3**). As dehydrogenases are only reductive in metabolically active cells, the consumption of MTS, monitored via colour change, is directly proportional to the relative number of metabolically active cells within a well.

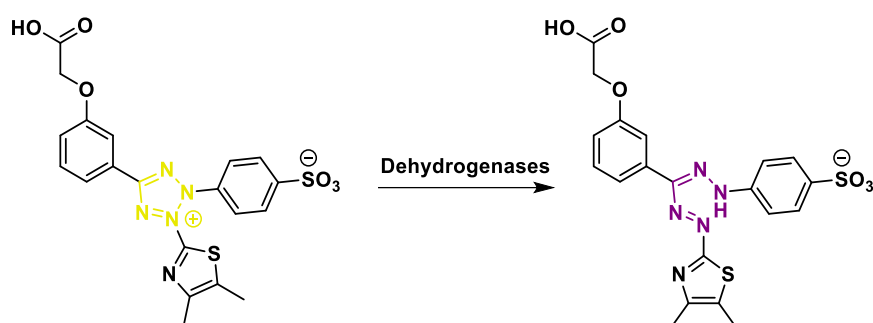


Figure 4.3: Reduction of MTS by dehydrogenase enzymes yielding a formazan product

The compounds were tested in two cell lines, HL-60 cells derived from an acute promyelocytic leukaemia patients, and A375 cells derived from a patient with malignant melanoma. The HL-60 cell line was chosen for consistency with previous experiments carried out in the group³⁶⁷, and A375 cells were chosen to evaluate the effect of the compounds on both a suspension (HL-60) and adherent (A375) cell line. Both experiments were carried out in the same manner.

The cells were treated with eight total concentrations of each compound over 72 hours. Upon treatment with MTS, dose-response curves were plotted, calculating IC_{50} values for each compound. Each concentration was tested in triplicate and the whole experiment for each ligand was repeated a total of three times. An example of typical dose-response curves calculated for two flexibly-linked regioisomers and two rigidly-linked regioisomers is presented below in **Figure 4.4**.

Dose-response curves were used to measure IC_{50} values for the active compounds, a summary of the results for both cell lines is presented **Figure 4.5**.

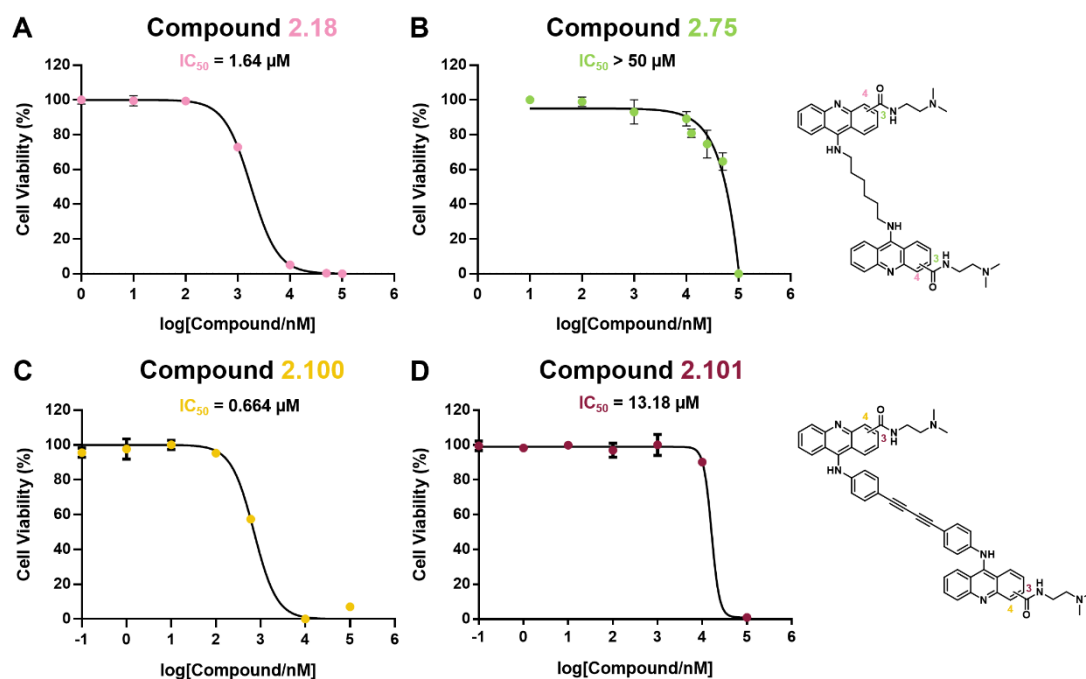


Figure 4.4: Dose response curves plotted after treatment of A375 cells with compounds **A. 2.18**, **B. 2.75**, **C. 2.100** and **D. 2.101** for 72 hours ($\pm$ SEM) ($n=3$). Plotted on GraphPad Prism.

In all cases the acridine-4-carboxamide dimers were more active than their 3-carboxamide regioisomers. The most potent compound was the C8-linked dimer, followed by two novel dimers, **2.109** and **2.105**. The 4-carboxamide rigidly linked dimers exhibit good anticancer activity but poor DNA binding, proposing the activity of these compounds is not directly linked to their binding properties. This suggests their activity is not related to the disruption of DNA processes by duplex DNA binding.

The disconnect between DNA binding and antitumour activity is further supported by the poor activity of the 3-carboxamide dimers **2.75** and **2.77**, which display good DNA binding in the FID assay. The loss of antitumour activity when relocating the carboxamide chain to position 3 is consistent with work reported by Denny, describing 9-aminoacridine-3-carboxamide inactive cancer cells,³⁵¹ mirrored in this work. Addition of a rigid linkers mildly increased the activity of 3-carboxamide dimers, yielding moderately potent compounds. The reduced activity of the acridine-3-carboxamide dimers may also relate to cell permeability.

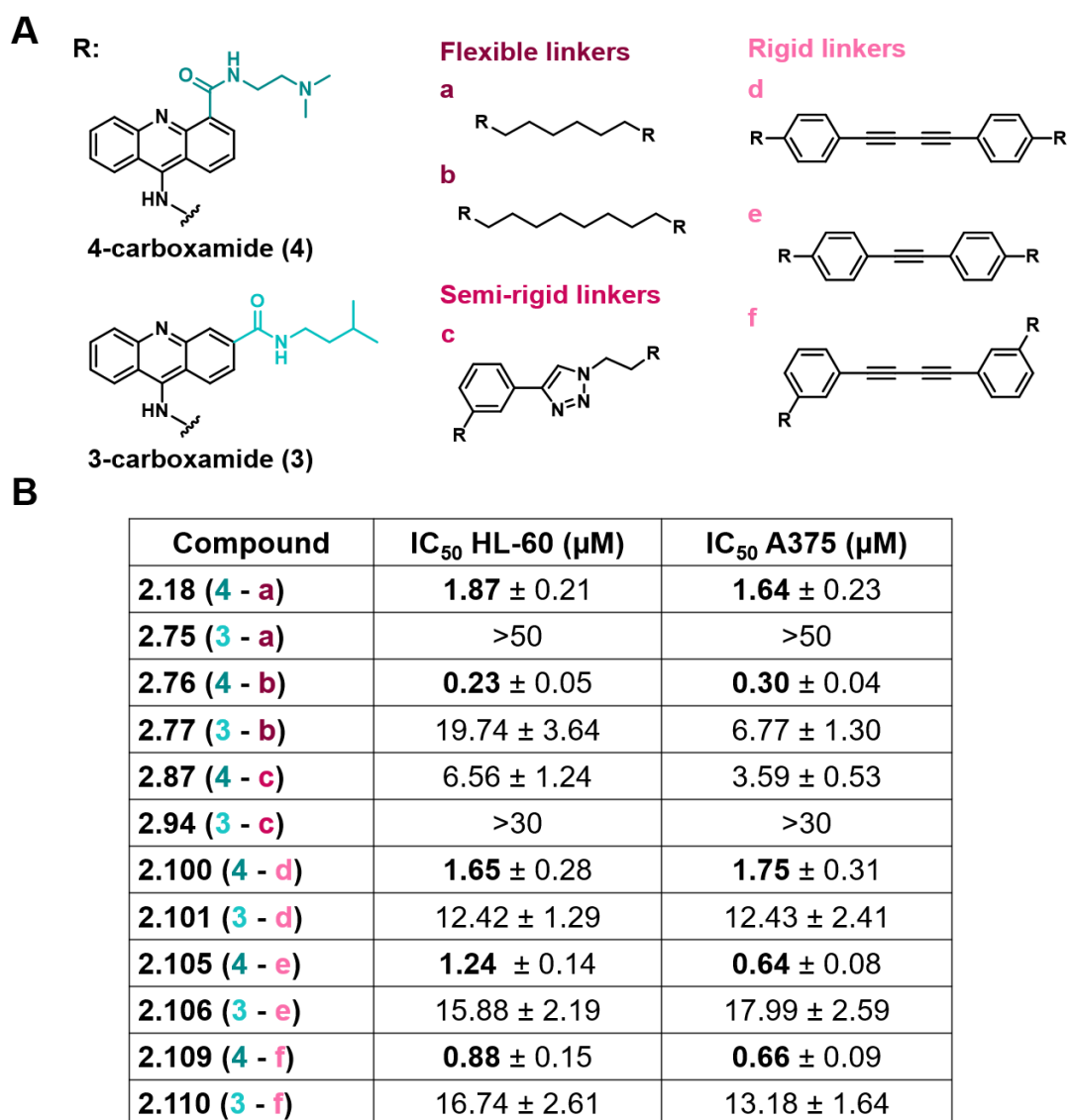


Figure 4.5: **A.** Chemical structures of the twelve synthesised acridine dimers. **B.** Table of IC₅₀ values calculated for each compound after treatment of HL-60 and A375 cells for 72 hours (±SEM). IC₅₀ values were calculated through GraphPad Prism fitted to a log(agonist) vs. response -- Variable slope (four parameters) model, $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X) * \text{HillSlope}})$ ($n=3$).

Acridine compounds have been well studied for their potent antitumour activity, with the 9-aminoacridide-4-carboxamides improving potency further.³⁵¹ The acridine compounds discussed in **section 2.1.4** have reported IC₅₀ values in HL-60 cells. The most potent compound reached an IC₅₀ value of 1.2 μM, an acridine-monomer “clicked” to an unsubstituted benzyl group. Novel acridine dimers **2.105** and **2.109** exhibit great anticancer activity, reaching nanomolar concentrations of 0.64 ± 0.08 μM and 0.66 ± 0.09 μM accordingly.

4.3.2 Synergism with DNA repair inhibitors

The MTS assays provide information on the impact of compounds on the proliferation and viability of the cells upon treatment. However, the mode of action of the active compounds remains unclear, with the exception of compound **2.18**, which has been previously reported as a potential topoisomerase II inhibitor³¹⁷. Considering the dimers' abilities to bind to four-way junctions, their mechanism of action could be linked to DNA repair mechanisms. A Holliday junction is formed during DNA repair via homologous recombination, if the compounds were to bind to this structure, preventing its resolution, this could result in cell death.

To investigate whether the dimers were involved in the DNA damage repair pathways, three compounds were taken forward for investigation of synergistic properties against MiaPaCa-2 cells treated with a known DNA damage repair inhibitor. The three compounds chosen were **2.76**, **2.100** and **2.101**. Compound **2.76** is the most potent acridine dimer of this series, therefore it was of interest to determine whether its activity could be further improved. Compound **2.100** is a novel compound presented in the thesis and was selected to evaluate the effects of flexibility restriction of the compound on cell viability. Finally, compound **2.101** was tested to investigate whether co-treatment with a DNA repair inhibitor would increase its cytotoxic effects, as well as directly compare the effect of the carboxamide relocation in these experiments.

The two selected inhibitors target proteins involved in DNA repair via both pathways, NHEJ and HR. NU7441 is a selective inhibitor of DNA-PK, involved in the detection and repair of DSBs via the NHEJ pathway, discussed in section 4.1.2.2⁵³¹. KU55933 is a selective inhibitor for ATM, a protein involved in DSB repair via HR, described in section 4.1.2.1^{532,533}. The chosen cell line was MiaPaCa-2, derived from a patient with pancreatic carcinoma. Approximately up to 25% of pancreatic cancers harbour defects in DNA damage response, leading to the investigation of treatment with inhibitors targeting key proteins in DNA repair, or co-treatment with DNA damaging agents^{534–536}. A total of six co-treatment combinations were tested, assessing the three compounds with both inhibitors.

MiaPaCa-2 cells were treated with six different concentrations of acridine dimer against six concentrations of a DNA repair inhibitor for 72 hours. Cell viability was measured by treatment of the plates with MTS.

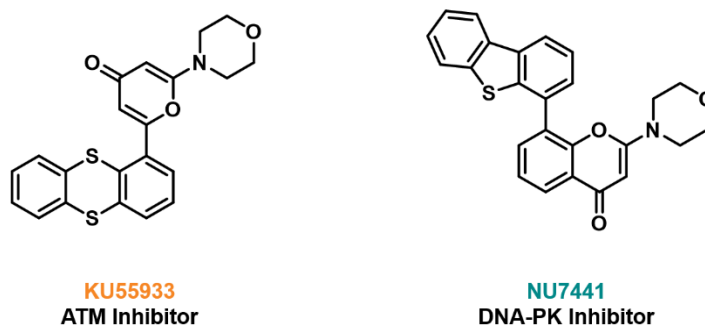


Figure 4.6: Chemical structures of ATM inhibitor KU55933 and DNA-PK inhibitor NU7441

IC₅₀ values in MiaPaCa-2 cells were recorded for all the participating compounds prior to co-treatment experiments.

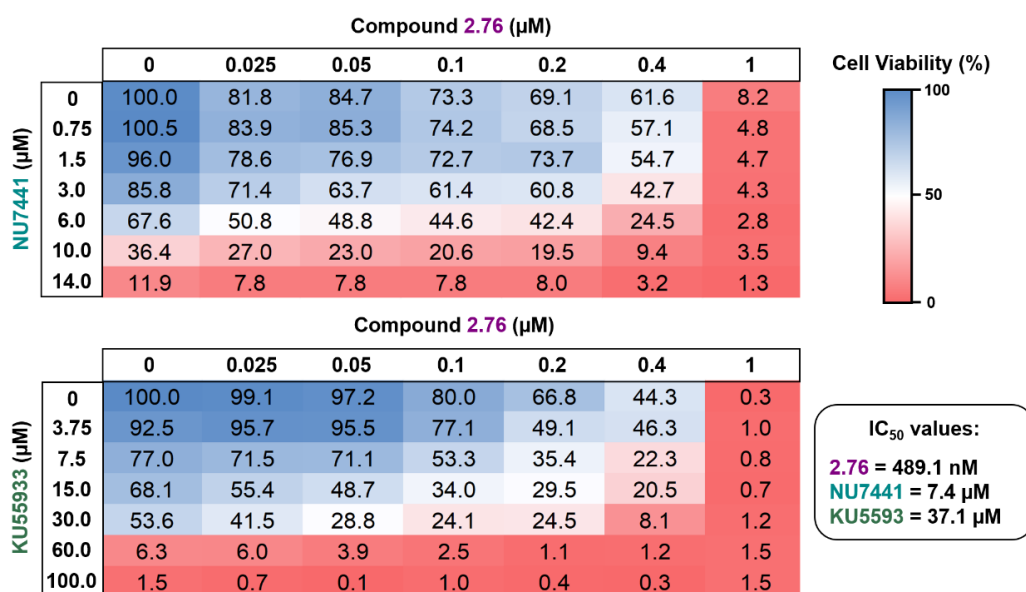


Figure 4.7: Cell viability of MiaPaCa-2 cells, co-treated with compound **2.76** and DNA repair inhibitors. Heat maps of cells treated with **2.76** from 1 μM (1:2 dilution) in combination with NU7441 from 14 μM and KU55933 from 100 μM for 72 hours at 1% DMSO final concentration. The data is presented as mean cell viability normalised to the vehicle control wells (n=3).

Compound **2.76** is a highly potent antitumour agent, therefore co-treatment with NU7441 and Ku55933 aimed to reduce the amount of compound necessary to have the same effect on the cells' viability. No obvious synergy is observed with the DNA-PK inhibitor. This acridine dimer was initially designed as a transcription inhibitor³¹⁷, however this mechanism of action has not been confirmed. Synergy is observed between the compound and KU55933. If **2.76** targets RNA polymerase, it may inhibit the transcription of key proteins involved in the NHEJ pathway, therefore leading to cell death when inhibiting the HR pathway too with KU55933. Treatment of the cells with 15 μM KU55933, a concentration that reduces cell viability to 70%, allows for a decrease to 50% with the addition of only 25 nM of **2.76**.

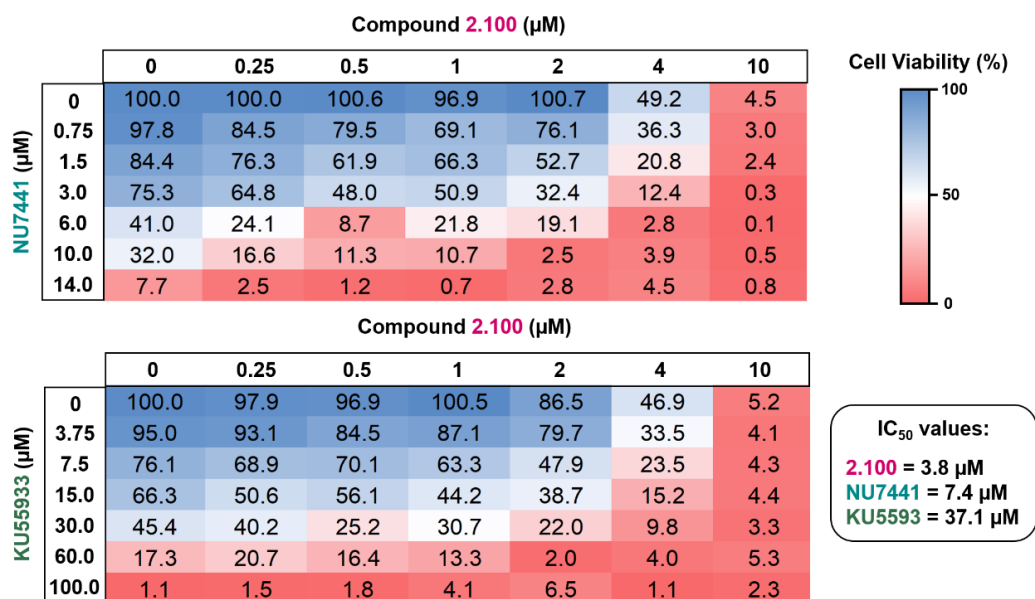


Figure 4.8: Cell viability of MiaPaCa-2 cells, co-treated with compound **2.100** and DNA repair inhibitors. Heat maps of cells treated with **2.100** from 10 μM in combination with NU7441 from 14 μM and KU55933 from 100 μM for 72 hours at 1% DMSO final concentration. The data is presented as mean cell viability normalised to the vehicle control wells (n=3).

Co-treatment of **2.100** with KU55399 and NU7441 indicate synergistic effects in MiaPaCa-2 cells. Co-treatment with both inhibitors at moderately cytotoxic concentrations (>50%) considerably increased the cytotoxicity of **2.100**, down to nanomolar concentrations. A higher synergistic interaction is observed with NU7441, suggesting **2.100** could disrupt the HR pathway, ultimately inhibiting both major DSB repair pathways, leading to cell death. KU55933 and **2.100** may be targeting different parts of the HR pathway, therefore when combined they act synergistically.

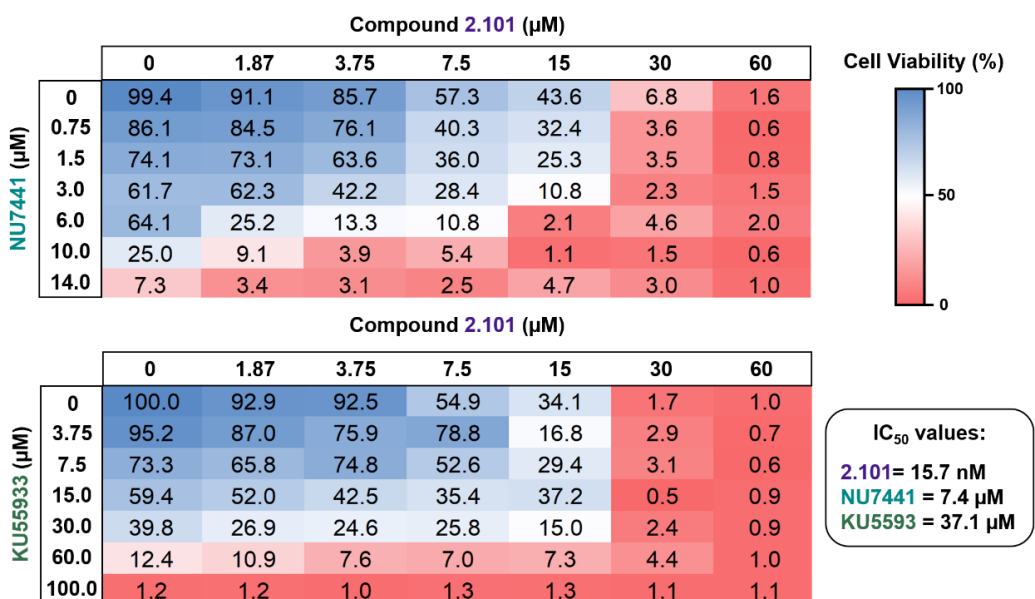


Figure 4.9: Cell viability of MiaPaCa-2 cells, co-treated with compound **2.101** and DNA repair inhibitors. Heat maps of cells treated with **2.101** from 1 μM (1:2 dilution) in combination with NU7441 from 14 μM and KU55933 from 100 μM for 72 hours at 1% DMSO final concentration. The data is presented as mean cell viability normalised to the vehicle control wells (n=3).

Co-treatment of compound **2.101** with NU7441 indicates synergy between the two compounds. The most pronounced change was observed when the cells were treated with 6 μ M of NU7441, reducing cell viability to 64%, which upon addition of 1.87 μ M of **2.101**, a drop to 25% was measured. No synergy is observed with KU55933, suggesting **2.101** could be causing HR inhibition.

4.3.3 Synergy data analysis

The data was analysed using a 4-parameter logistics (4PL) curve, a mathematical model that presents cell viability data in a sigmoidal shape. This model was selected for GraphPad prism analysis of the data presented in **Figure 4.4**. This model was also used for further analysis of the synergy experiments. The formula used for drug 1 with dose D_1 for the 4PL model is:

Equation 4.1: 4-Parameter logistic model synergy equation

$$R_1(D_1) = R_{\min,1} + \frac{R_{\max,1} - R_{\min,1}}{1 + \left(\frac{D_1}{IC_{50,1}}\right)^{+b_1}}$$

In this equation R_1 = the response of drug 1, $R_{\min,1}$ = the minimum response of drug 1, $R_{\max,1}$ = the maximum response of drug, $IC_{50,1}$ = the IC_{50} value recorded of drug 1 and b_1 = the slope.

When using this equation for synergy analysis $R_1(D_1)$ is replaced by:

Equation 4.2: Response indicates drug 1 and 2 combination response

$$R = R_{comb}(D_1, D_2)$$

R represents the response measured by the combination of drug 1 at D_1 with drug 2 at D_2 . **Equation 4.1** is subsequently rearranged to solve for the predicted dose of drug 1 ($D_{x,1}$) required in isolation to gain the same response R measured by the combined concentrations of D_1 and D_2 . The rearranged equation results in:

Equation 4.3: Rearranged formula of Equation 4.1

$$D_{x,1}(R) = IC_{50,1} \cdot \left(\frac{R_{\max,1} - R_{\min,1}}{R - R_{\min,1}} - 1 \right)^{\frac{1}{b_1}}$$

This equation is carried out twice, once for drug 1 ($D_{x,1}$) and once for drug 2 ($D_{x,2}$). Upon calculation of the predicted doses, they can be used in the Loewe Additivity equation:

Equation 4.4: Combination index equation based on the Loewe additivity model

$$CI(R) = \left(\frac{D_1}{D_{x,1}(R)} \right) + \left(\frac{D_2}{D_{x,2}(R)} \right)$$

CI represents the combination index, a score that describes the degree of synergy, if present, between two drugs. An additive effect describes a response caused by two drugs equally contributed, therefore D_1 would be responsible for 50% of the cell response and D_2 the remaining 50%. As a result, the CI calculated would equate to 1, as the predicted doses of the drugs would be double the actual doses. If the two drugs work synergistically, the actual doses used to cause a response would equate to lower than 50% of the predicted doses, and the CI would score below 1. In contrast, an antagonistic effect would be described as a CI score greater than 1, with higher doses of the drugs required in combination to each other to gain the same effect as in isolation. The Loewe additivity model used for the analysis assumes the two drugs are acting on the same target or pathway.

The six combinations presented in **section 4.3.2** between the acridine dimers and DNA repair inhibitors were analysed using this method. A CI score was calculated for each combination carried out between the two compounds in each co-treatment. The most promising results were afforded by the combination of compound **2.100** and the NU7441 inhibitor. A heatmap of the CI score measured is presented in **Figure 4.10**. The data was also plotted in an isobologram, where the dotted linear line represents an additive effect. Any CI scores to the left of the line are located in the synergy region and any on the right in the antagonistic region.

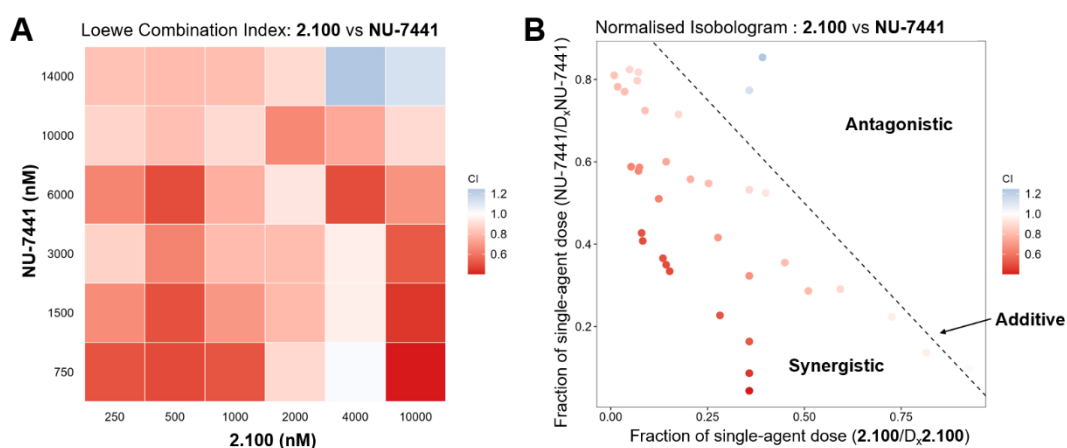


Figure 4.10: **A.** A heatmap of the combination index scores calculated from the co-treatment of **2.100** with NU7441 in MiaPaCa-2 cells, **B.** An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

The scores calculated when the cells were treated with compound **2.100** and NU7441 show good synergy, with the CI scores all placed in the synergistic region of the

isobologram, as low as 0.50. Two CI scores are located in the antagonistic region, however they are measured at the highest concentrations of both compounds, which are able to reduce cell viability to 0% in isolation, therefore they were not considered.

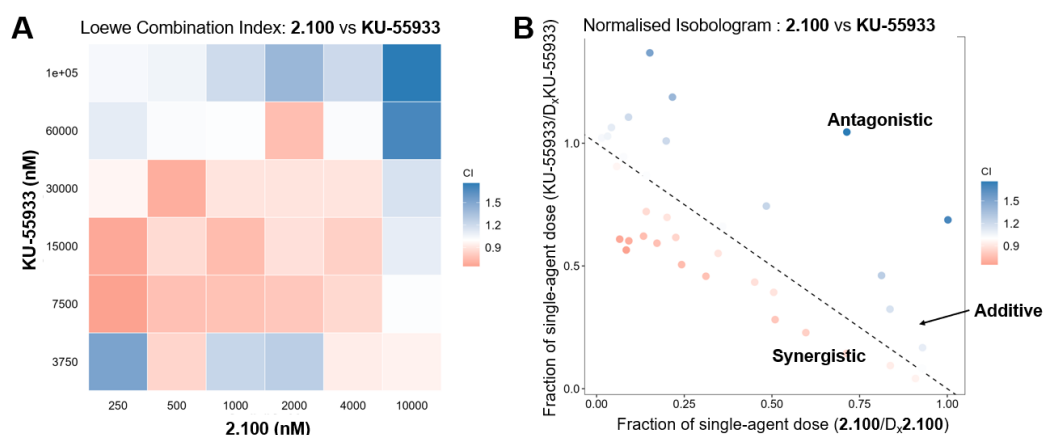


Figure 4.11: **A.** A heatmap of the combination index scores calculated from the co-treatment of **2.100** with KU55933 in MiaPaCa-2 cells, **B.** An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

In comparison, when cells were treated with **2.100** and KU55933 the results indicate largely an additive effect, with most CI scores calculated around 1. However, small levels of synergy are observed when cells were treated with $\leq 1 \mu\text{M}$ **2.100** and $\leq 30 \mu\text{M}$ of KU55933, causing a greater effect on cell viability than in isolation. Further experiments should be undertaken focusing on these concentrations to confirm these preliminary results, that agree with the heatmap data presented in **Figure 4.8**.

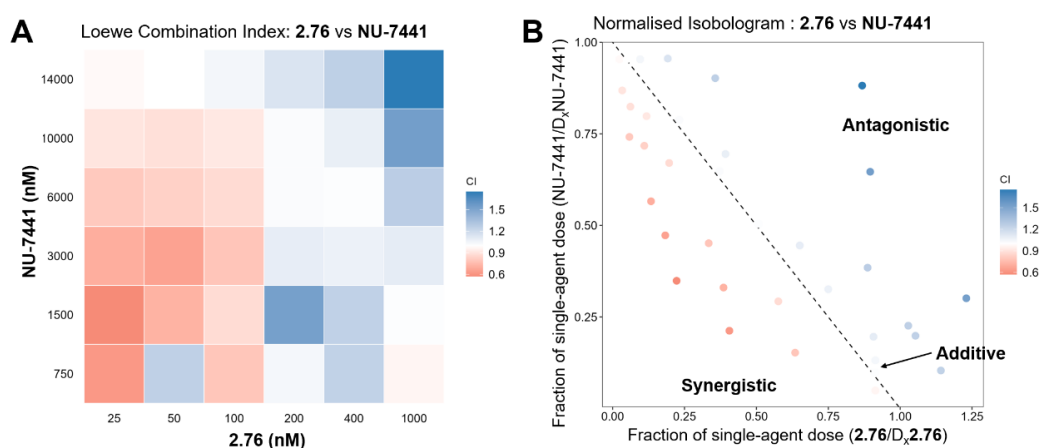


Figure 4.12: **A.** A heatmap of the combination index scores calculated from the co-treatment of **2.76** with NU7441 in MiaPaCa-2 cells, **B.** An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

The most potent acridine dimer evaluated was **2.76**, when cells were co-treated with NU7441 synergistic effects were measured with CI scores of 0.6 reached with only 25 nM of **2.76**. The majority of the remaining CI scores are near 1, suggesting further experiments could validate the degree of the synergism.

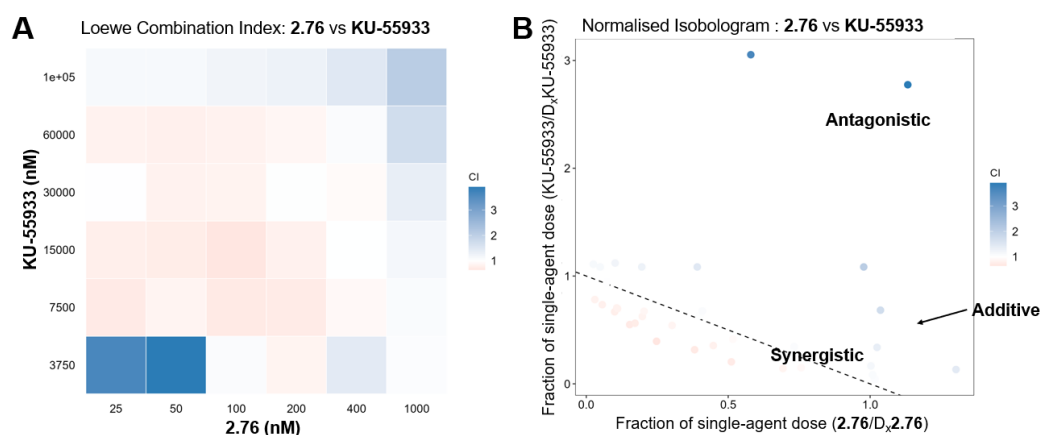


Figure 4.13: **A.** A heatmap of the combination index scores calculated from the co-treatment of **2.76** with KU55933 in MiaPaCa-2 cells, **B.** An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

When combining **2.76** with KU55933 an additive effect is observed, with CI scores located near the 1 mark. This result is expected if **2.76** is acting on the HR pathway, therefore when combined with a HR inhibitor no greater effect is observed.

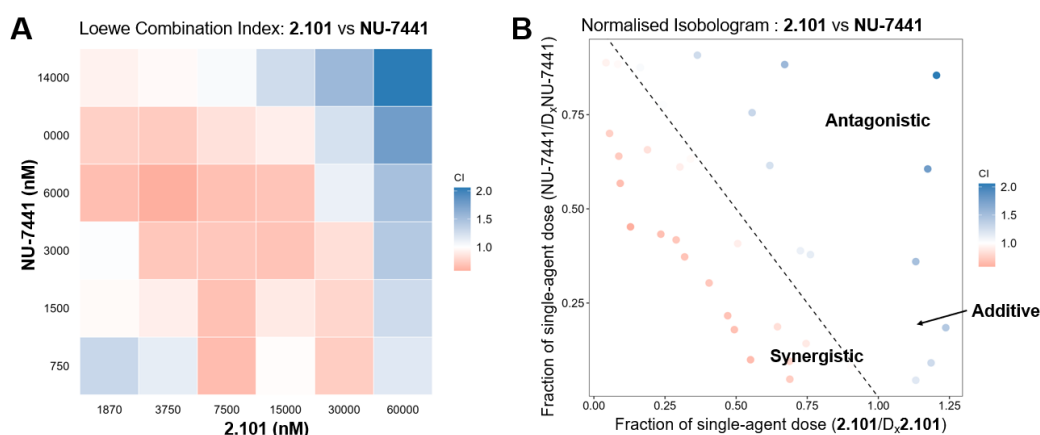


Figure 4.14: **A.** A heatmap of the combination index scores calculated from the co-treatment of **2.101** with NU7441 in MiaPaCa-2 cells, **B.** An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

Agreeing with the data presented in **Figure 4.9**, co-treatment of cells with **2.101** and NU7441 indicates a level of synergy between the two compounds. Moderate CI scores of 0.8 are measured with treatment of $\leq 7.5 \mu\text{M}$ **2.101** and $\leq 60 \mu\text{M}$ of NU7441 showing some promise.

No synergy is observed upon co-treatment of cells with **2.101** and KU55933, suggesting an antagonistic effect occurring between the two compounds with CI over 1. If both compounds are acting on the HR pathway, they could be competing leading to antagonism.

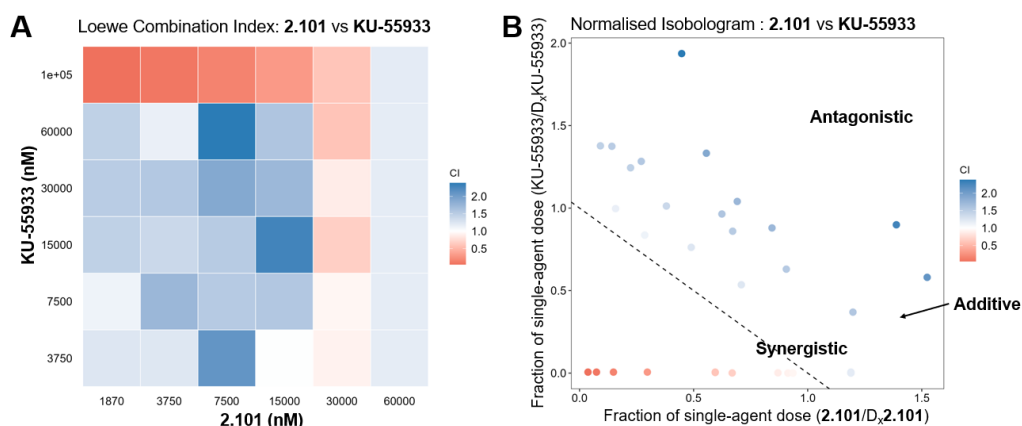


Figure 4.15: A. A heatmap of the combination index scores calculated from the co-treatment of **2.101** with KU55933 in MiaPaCa-2 cells, B. An isobologram highlighting the synergistic, additive and antagonistic regions ($n=3$).

The experiments presented provide a good starting point to encourage further investigation into the synergistic effects observed. Acridine dimers exhibit good activity upon co-treatment with DNA repair inhibitors, potentially leading to new therapeutic treatments for cancer. However, cell viability assays provide limited information on the mechanism of action of small molecules in cells, therefore further experiments to understand their mechanisms are required to fully interpret these preliminary results.

4.3.4 Cell viability assay – Acridine trimers

The effect of the acridine trimers on cell viability was investigated in HL-60 and MiaPaCa-2 cells. The cells were treated for 72 hours, followed by treatment with MTS. Dose-response curves were plotted and IC_{50} values were calculated.

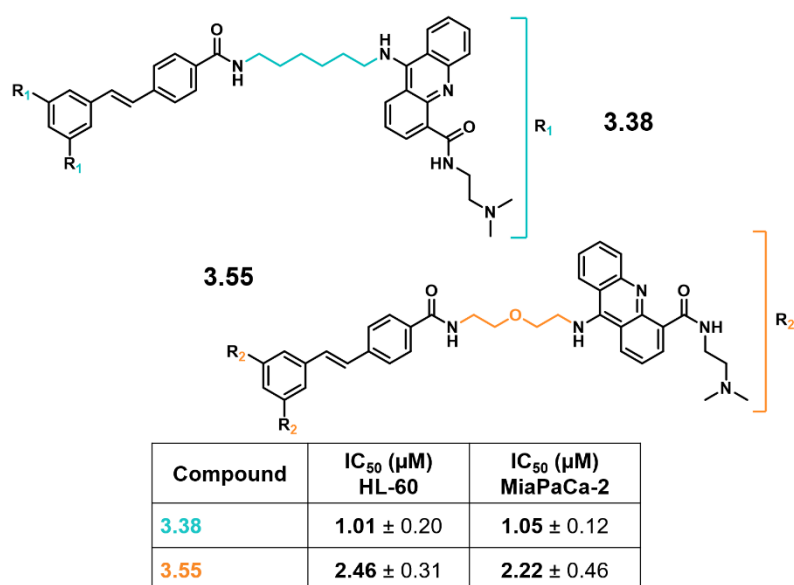


Figure 4.16: Cell viability of HL-60 and MiaPaCa-2 cells upon treatment with **3.38** and **3.55** for 72 hours. IC_{50} values were calculated from dose-response curves plotted in GraphPad Prism ($n=3$).

Both trimers exhibit potent antitumour activity in HL-60 and MiaPaCa-2 cells. Further studies are required to discover the mechanism of action of these compounds, however they exhibit good potential for DNA junction targeting antitumour agents. An ongoing collaboration with Dr Sebastien Britton at the University of Toulouse suggests compound **3.55** acts as a topoisomerase II inhibitor.

4.4 Conclusions

Defects in the DNA damage response pathways have been identified in several types of cancers. Developing small molecules that can inhibit the remaining active DNA repair pathways takes advantage of this property, incorporating selective targeting of cancer cells over healthy cells. A major pathway in DSB repairs, the deadliest form of DNA damage, is homologous recombination. A series of acridine dimers were designed to target a higher order intermediate DNA structure that forms in this process, the Holliday junction.

The cytotoxicity of the synthesised dimers was assessed on two cells lines, HL-60 and A375 cells, highlighting several potent small molecules. The cytotoxicity of the compounds was directly related to the position of the threading carboxamide side chain, with relocation to position 3 significantly reducing the antitumour activity. Furthermore, the addition of the rigid linkers yielded potent antitumour agents with nanomolar range activity.

Co-treatment of three compounds was explored with DNA repair inhibitors, to investigate synergy. Promising results were collected, indicating potential for the design of combination therapies with a DNA repair inhibitor and acridine dimers. The cells became more sensitive to the compounds in the presence of the inhibitors, suggesting greater potency may be observed if tested in cancer cells defective in DNA repair pathways.

Chapter 5

Conclusions and Perspective

The genetic information of a cell is stored in molecules of deoxyribonucleic acid (DNA), consisting of two polynucleotide chains, coiled around each other in a right-hand turn, called B-form DNA. However, DNA can adopt a variety of alternative conformations based on sequence motifs and protein interactions, referred to as non-B-DNA structures. The four secondary DNA structures most commonly targeted with small molecules for the development of therapeutic treatments are G-Quadruplexes, i-motifs, three-way junctions (3WJs) and four-way junctions (4WJs).

A three-way junction consists of three duplex DNA sections connected at a single point. They can form in the presence of direct repeats in the genome, and have been extensively associated with trinucleotide repeats, linked to over 40 degenerative human disorders.^{209,215–220,537} A four-way junction is made up of four duplex DNA sequences, and can exist in two conformations, the open and X-stacked conformation.³⁷⁹ The 4WJ can form in the presence of inverted repeat sequences, making up 1.6% of the genome.²¹² Additionally, it is also commonly referred to as a Holliday junction (HJ),²²⁹ as it is a structural intermediate in homologous recombination (HR), a DNA repair pathway responsible for the repair of double strand breaks (DSBs) in DNA.

DNA damage response pathways are often defective in cancer cells, providing a therapeutic opportunity. Targeting the 4WJ (HJ) intermediate in HR with small molecules could provide a therapeutic approach for selective targeting of cancer cells over healthy cells.

A crystal structure of an *N,N'*-dimethylaminoethyl 9-aminoacridine-4-carboxamide dimer linked through a flexible methylene chain, bound in the central pocket of a 4WJ through a novel mechanism (**Figure 5.1**), was reported by the Searcey group in collaboration with the Cardin group.³¹⁹ This compound was initially designed to bisintercalate into duplex DNA, however, a second crystal structure was published presenting the dimer bound to two duplex DNA simultaneously, carrying out duplex-to-duplex DNA crosslinking (**Figure 5.1**).³³⁸

The two binding modes were combined to design a series of novel acridine dimers that could potentially bind across a 4WJ, trapping the formation by simultaneously intercalating into two of the junction arms or via binding in the centre of the 4WJ.

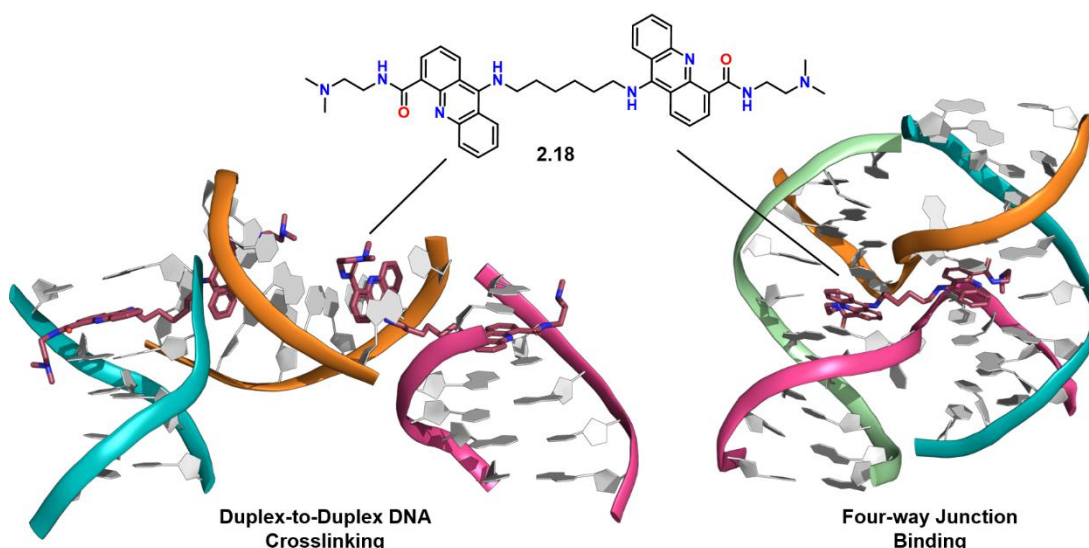


Figure 5.1: Crystal structures of 9-aminoacridine-4-carboxamide dimer **2.18** carrying out duplex-to-duplex DNA crosslinking (PDB **2GB9**), and bound in the centre of a 4WJ (PDB **2GWA**)

A total of twelve dimers were synthesised (**Figure 5.2**), covering four-flexibly linked compounds, bearing methylene linkers. These were complemented by two semi-rigidly linked dimers, prepared via “click” chemistry. Finally, six novel compounds bearing rigid linkers, comprised of alkyne and benzene groups, were prepared, completing the set. The compounds were evaluated in a series of biophysical assays, investigating their binding to DNA junctions, their effect on junction stability and selectivity over duplex DNA.

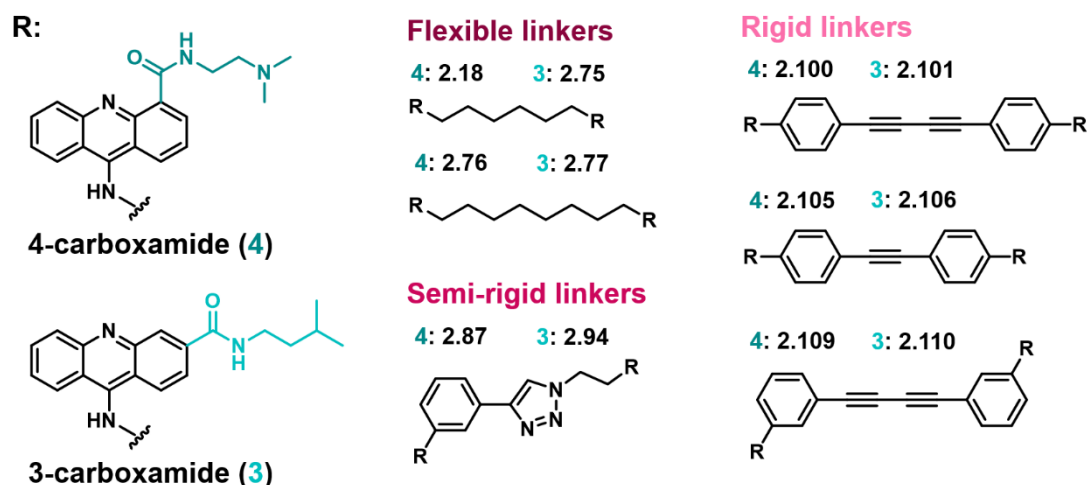


Figure 5.2: A series of 9-aminoacridine-3/4-carboxamide dimers prepared, varying in linker flexibility

The four flexibly-linked dimers **2.18**, **2.75**, **2.76** and **2.77**, exhibited similar behaviours, displaying strong DNA binding, monitored in a fluorescent indicator displacement (FID) assay, further supported by their ability to carry out duplex-to-duplex DNA crosslinking, measured using a gold-nanoparticle-based assay. Binding to 4WJs was observed in EMSA experiments down to 1.56 μM , whilst all four dimers were able to

induce the formation of the 4WJ at room temperature at similar concentrations. A FRET assay observing 4WJ conformation, demonstrated nanomolar concentrations induced a conformation change to the 4WJ, indicating the dimers are unable to bind to the open form. High stability of DNA junctions was measured in a melting experiment upon binding of the dimers (ΔT_m up to 45 °C for the 4WJ at 10 μ M), however little selectivity was observed upon addition of other secondary DNA structures.

The semi-rigid dimers **2.87** and **2.94** exhibited duplex-to-duplex DNA crosslinking with mild DNA binding measured in the FID assay. Binding to the 4WJ was displayed at concentrations higher than 25 μ M, however only the 3-carboxamide regioisomer **2.94** was able to induce the assembly of the 4WJ at room temperature. Good stabilisation of the 4WJ (ΔT_m = 11 °C, 10 μ M) was recorded, minimally affected by the addition of DNA competitors, indicating 4WJ selectivity.

The 3-carboxamide rigidly-linked dimers **2.101**, **2.106** and **2.110**, displayed poor DNA binding, no stabilisation of the duplex DNA structure in the melting experiments, requiring higher concentrations than the rest of the dimers to carry out duplex-to-duplex DNA crosslinking. Poor stabilisation of the DNA junctions was observed, whilst gel electrophoresis experiments highlighted 4WJ binding at concentrations higher than 200 μ M, and a conformational change was recorded at 50 μ M.

The shift of the carboxamide chain to position 4, in compounds **2.100**, **2.105** and **2.109** had a significant impact on the DNA binding abilities of the dimers bearing rigid linkers. In the FID assay, the compounds exhibited poor DNA binding, but could crosslink duplex-to-duplex DNA, suggested the simultaneous binding of both acridine moieties provided increased stability, a property agreeing with the intended binding mode of the dimers to 4WJs. The dimers were able to bind to pre-formed 4WJs at concentrations higher than 200 μ M, however, when allowed to direct junction assembly, binding was measured down to 25 μ M, for compounds **2.100** and **2.105**. These results suggest that perhaps a different binding mode is carried out upon binding to a pre-folded 4WJ, compared to ligand-induced 4WJ formation. This hypothesis is further supported by the minimal 4WJ stabilisation recorded by these compounds in the melting assay.

The introduction of rigid linkers to the acridine dimers holds promise as a modification to improve DNA junction selectivity over duplex DNA. The compounds show weak binding to duplex DNA whilst bearing two DNA intercalating groups, confirming linker variation can be used to alter the dimers DNA binding properties. However, further improvement of the structures is necessary to lower the binding concentrations to

DNA junctions. An unknown factor when targeting DNA junctions with acridine dimers is the optimal linker length, the design of new linkers adjusting length whilst maintaining the rigidity could lead to the identification of a compound combining the strong 4WJ binding and stabilisation of the flexibly-linked dimers and the weak duplex DNA binding of the rigidly-linked dimers.

The cytotoxicity of the acridine dimers was assessed in leukaemia (HL-60) and melanoma (A375) cancer cell lines, highlighting several potent small molecules. The potency of the compounds is directly linked to the position of the *N,N'*-dimethylaminoethyl carboxamide chain, with the 4-carboxamide dimers outperforming their 3-carboxamide regioisomers. IC₅₀ values of 1.65 µM (**2.100**), 648 nM (**2.105**) and 664 nM (**2.109**) were recorded for the rigidly linked dimers, showing promise as potent antitumour agents.

Synthetic lethality has been demonstrated as a successful cancer treatment, with the administration of PARP inhibitors to BRCA1/2 deficient cancer patients proving one of the most well-known cases.^{256,257} PARP inhibitors inhibit the repair of single strand breaks in the DNA, evolving into DSBs, unable to be repaired via the HR pathways due to the BRCA1/2 deficiency. A strategy to apply this treatment to non-deficient cancer types could be based on the chemical induction of synthetic lethality. If the acridine dimers act via inhibition of the HR pathway through HJ binding, the combination with DNA repair inhibitors could lead to a more potent effect in the cells. This hypothesis was explored in pancreatic cancer cells (MiaPaCa-2) with the combination of compounds **2.76**, **2.100** and **2.101**, with DNA repair inhibitors NU7441 and KU55933, that act on the non-homologous end joining and homologous recombination pathways, respectively. Co-treatment of **2.100** with NU7441 indicated the two compounds would act synergistically, measuring considerably higher cytotoxicity in the cells when combined.

The preliminary data gathered would require follow-up experiments, investigating the exact mechanism of action of compound **2.100**, however, the results presented here provide a promising starting point. Carrying out a synergy evaluation assay with an Rad51 inhibitor would compliment these results, as Rad51i acts on a different stage of the HR pathway, confirming whether the dimers are involved in this process. Additionally, as the acridine dimers bind to DNA, a DNA damage assay would be of interest, as it would assess whether the mechanism of action of the compounds is associated with DNA damage. The synergy experiments could be performed with co-

treatment of PARP inhibitors, as they have been studied alongside DNA damaging agents and shown to act synergistically.

Two novel tri-acridine compounds were designed to target three-way junctions, aimed to mimic the structure of the junction. The final compounds prepared consisted of a central 1,3,5-tristyrylbenzene core, extending symmetrically towards three directions, bearing *N,N*-dimethylaminoethyl 9-aminoacridine-4-carboxamide groups at each extremity (**Figure 5.3**).

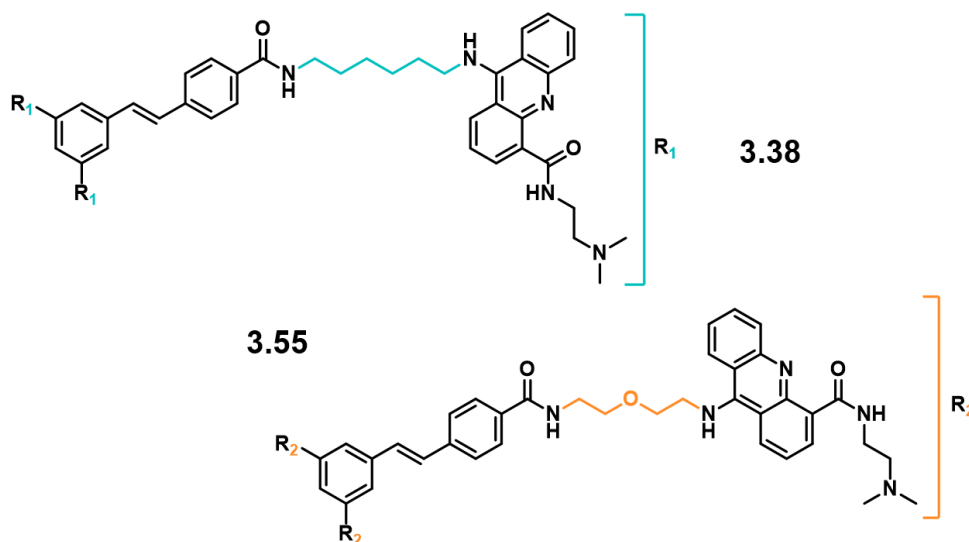


Figure 5.3: Design of two *N,N*-dimethylaminoethyl 9-aminoacridine-4-carboxamide trimers, linked to a 1,3,5-tristyrylbenzene core

The design strategy is based on the central core located on top of the central pocket of a 3WJ, with the three acridine arms extending out and intercalating into three duplex DNA arms of the junction.

Trimers **3.38** and **3.55** were able to crosslink DNA, confirming they could bind simultaneous duplex DNA molecules, a requirement for the intended 3WJ binding mode. Gel electrophoresis experiments demonstrated that both trimers were able to induce the formation of a 3WJ at nanomolar concentrations ($< 1.56 \mu\text{M}$). Upon repeating the experiment with a 4WJ, the gel indicated no binding to the junction by the trimers. High stabilisation of a 3WJ was observed in melting experiments, measuring a $\Delta T_m = 30^\circ\text{C}$ increase upon binding of the trimers ($10 \mu\text{M}$). Upon addition of a duplex DNA competitor, a small drop was observed ($\Delta T_m = 18^\circ\text{C}$), indicating a preference for 3WJ binding.

The cytotoxicity of the acridine trimers was assessed in leukaemia (HL-60) and pancreatic (MiaPaCa-2) cancer cells. High potency was recorded for both compounds, with IC_{50} values of $1.01 \mu\text{M}$ for **3.38** and $2.22 \mu\text{M}$ for **3.55**.

These results validate the design of the acridine trimers to target three-way junctions via mimicking the DNA structure. The incorporation of a rigid central unit of trigonal shape extended via a simple linker bound to 9-aminoacridine-4-carboxamide groups at the end resulting in the design of two novel 3WJ binders. Whilst **3.18** and **3.55** induce 3WJ assembly at nanomolar concentrations and stabilise 3WJs by 32 °C, some improvements could be made in their 3WJ selectivity. A clear preference for 3WJs was noted in the melting competition assay, however, the compounds are still able to bind and stabilise 4WJs and duplex DNA to a lesser extent. Modification of the linker connecting the central group and the intercalating units, altering length and flexibility, could potentially improve the design even more. Additionally, further cellular studies would be of interest, carrying out synergy experiments with DNA repair inhibitors, explored with the acridine dimers, evaluating the trimers.

Finally, the promise of the trimer design could be adapted to target four-way junctions with acridine tetramers, maintaining a similar design. Instead of extending out of the central benzene ring at positions 1', 3' and 5', the arms could be extended off positions 1', 2', 4' and 5'. The design would result in a symmetrical tetrameric compound made of a rigid central unit extending out of four positions linked to 9-aminoacridine-4-carboxamide through a simple linker.

Chapter 6

Experimental

6.1 General procedures

6.1.1 Reagents and solvents

All chemicals were purchased from Merck, Fisher Scientific or Fluorochem. Black low binding 96 well plates for the FID and FRET assays were purchased from Fisher Scientific. Strip tubes for the melting assays were purchased from QIAGEN. MTS was purchased from Promega. Pre-made 10% TBE gels and x10 TBE buffer were purchased from Invitrogen. Oligonucleotides were purchased from Eurogentec. Calf Thymus DNA was purchased from Merck, diluted to a 1 mg/mL solution in water and stored at 4 °C. Ethidium bromide was purchased from Fisher Scientific as a 10 mg/mL solution. All water used was distilled.

6.1.2 Instrumental techniques

All ^1H , ^{13}C and ^{19}F NMR were collected using a Bruker spectrometer operating at 400 MHz (^1H), 100 MHz (^{13}C) or 376 MHz (^{19}F), using the specified deuterated solvent. Chemical shifts were recorded in ppm, referenced to residual solvent peak (for ^1H NMR) of CHCl_3 (7.26 ppm), MeOH (3.31 ppm), DMSO (2.50 ppm) and H_2O (4.79 ppm). The multiplicities in the NMR spectra are described as s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet and br = broad, with coupling constants reported in Hz.

High resolution masses were recorded using QExactive mass spectrometer (Thermo) using its Vanquish UHPLC using electrospray ionisation (ESI). Low resolution mass was recorded using an Agilent 1200 Series equipped with a 6460 triple quadrupole.

IR was performed using a Perkin-Elmer Spectrum BX FT-IR spectrometer and analysed using Spectrum v5.3.1 software.

Analytical RP-HPLC was performed on an Agilent 1200 using an Agilent eclipse XDB-C18 column, 4.6 x 150 mm, 5 μM and a flow rate of 1 mL/min. Solvent A = H_2O + 0.1% TEA and Solvent B = MeOH + 0.1% TEA. Gradient 5% B up to 95% B over 20 minutes. Detection wavelengths were 214 nm, 254 nm and 450 nm.

6.2 Biological assays

6.2.1 Fluorescent indicator displacement (FID) assay

A calf-thymus DNA (ctDNA) solution was prepared in milliQ water to a final concentration of 88 μM and an ethidium bromide solution in 0.1 M Tris, 0.1 M NaCl, pH 7.0 to a final concentration of 4.4 μM . To each well of a 96-well black low binding plate, 88 μL of the EtBr solution was added, 10 μL of ctDNA and 2 μL of compound in increasing concentrations between 10 mM – 0.1 μM (final concentrations 200 μM – 2 nM). The plate was incubated for 30 minutes at room temperature, after which it was read on a BMG Labtech POLARstar OPTIMA microplate reader (excitation 560 nm, emission 590 nm). The experiments were performed in triplicate, each plate included six control wells, 3 containing no drugs, equating to 100% fluorescence, and 3 containing no DNA, equating to 0% fluorescence. The fluorescence readings are reports as % of fluorescence relative to the controls, C_{50} values were calculated using GraphPad Prism version 8.0 software.

6.2.2 UV absorbance screen

10 μM solutions of each compound in water were analysed using UV absorbance spectroscopy between 300 and 650 nm using a quartz cuvette with a 1 cm path length in a Hitachi U-3000 Spectrophotometer.

6.2.3 UV titration

A 1 μL addition of compound at 10 mM was carried out a total of 10 times (1 – 100 μM) into 1 mL of water in a quartz cuvette with a 1 cm path length. The UV absorbance was recorded between 350 and 600 nm using a Hitachi U-3000 Spectrophotometer.

6.2.4 Gold nanoparticle-based assay

6.2.4.1 Synthesis of gold nanoparticles

The nanoparticles were synthesised via the citrate reduction reported by Enüstün and Turkevich³⁷⁵. Two separate solutions of gold (III) chloride trihydrate (12.5 mg, 32 μmol in 100 mL of distilled water) and trisodium citrate (50 mg, 158 μmol , in 50 mL of distilled water) were prepared. The two solutions were heated to 60 °C and allowed to stir for 5 minutes. The sodium citrate solution was rapidly poured into the gold

solution whilst stirring vigorously. The combined solution was heated to 85 °C and left to stir for 2.5 hours. The solution was allowed to cool to room temperature and a red clear AuNPs was obtained. The nanoparticles were filtered through a Miller GP syringe driven filter unit (0.22 µm), and the nanoparticles were stored in the fridge until use.

6.2.4.2 Preparation of the dsDNA

The sequences picked for the AuNP assay were those reported in the paper published by Marin *et al*³⁶⁸. Two complementary 35 base pair oligonucleotides were purchased from Eurogentec. One of the oligonucleotides was requested with a thiol group at the 5' position, for the attachment of the DNA to the gold nanoparticles. The sequences of the oligonucleotides are described below:

S1: 5' - [ThiC6] - CTACGTGGACCTGGAGAGAGGAAGGAGACTGCCTG – 3'

S2: 3' – GATGCACCTGGACCTCTCTCCTTCCTCTGACGGAC – 5'

Stock solutions of 50 µM were prepared for each oligonucleotide in 5 mM HEPES and 10 mM trisodium citrate buffer. For the annealing, 25 µL of each stock solution were combined in an Eppendorf tube and heated to 100 °C and allowed to cool to room temperature overnight, which was stored at -80 °C until needed.

6.2.4.3 Synthesis of gold nanoparticles functionalised with thiolated dsDNA

Annealed thiolated dsDNA (31.5 µL of a 25 µM solution in trisodium citrate and HEPES) was added to 1.5 mL of gold nanoparticles. The solution was mixed with a pipette and allowed to stand for 1 minute. Trisodium citrate (30 µL of a 500 µM solution, pH 3.0) was added to the AuNP solution, it was mixed up and down with a pipette and left to stand for 10 minutes. A small amount of NaCl (52.5 µL of a 2 M solution) was added to the sample. It was quickly mixed with a pipette and mixed on a rotary mixer for 20 minutes. More NaCl (150 µL) was added to the sample, it was mixed with a pipette swiftly and mixed on a rotary mixed for 40 minutes. The solution remained a clear red throughout, confirming no aggregation had occurred. The sample was centrifuges for 30 minutes at 8,000 rpm in an Allegra™ X-22R centrifuge, Beckman Coulter. The supernatant was removed and the nanoparticles left at the bottom of the tube were washed with PB (1.5 mL of 10 mM, pH 7.4) and centrifuged again. This step was repeated a total of three times. In the last step, after the supernatant was removed, the centrifuged nanoparticles were resuspended in 1.5 mL of PB and stored in the fridge until required, maintaining their initial concentration.

6.2.4.4 Titration of dsDNA functionalised AuNPs with compounds

Stock solutions of 1 mM were prepared for all compounds tested and 9-aminoacridine. To a sample of dsDNA functionalised AuNPs (570 μ L), increasing concentrations of compound were added. A total of 10 additions (1 μ L of 1 mM stock solution added each time) were completed (total volume of 580 μ L), with concentrations ranging from 0 to 17.2 μ M. The samples were shaken by hand for 1 minute between each addition, and UV-Vis extinction recordings were measured before and after each addition of the compounds. Similarly, the procedure was repeated using 9-aminoacridine, adding 2 μ L of 1 mM stock solution each time, therefore reaching a total volume of 590 μ L. Ultraviolet-visible (UV-Vis) spectra were recorded using a Hitachi U-3000 spectrophotometer at room temperature. Quartz cuvettes with a 1 cm path length were used.

6.2.5 EMSA Four-Way DNA Junction Binding Assay

The oligonucleotides used in the 4WJ binding assays are:

B = 5' – GCCTAGCATGATACTGCTACCG - 3'

H = 5' – CGGTAGCAGTACCGTTGGTGGC - 3'

R = 5' - GCCACCAACGGCGTCAACTGCC - 3'

X = 5' – GCCAGTTGACGTCATGCTAGCC - 3'

6.2.5.1 4WJ trapping experiment

A 1.25 μ M solution of stoichiometric amounts of oligonucleotides X, R, H and B was prepared in Tris borate pH 8.0 buffer. A total of eight concentrations were tested for each compound in 1 in 2 dilutions prepared in separate eppendorf tubes, each containing 50 μ L of the DNA solution and 1 μ L of compound (x50 of the final concentration). The tubes were heated to 90 °C and allowed to cool down overnight. 1 μ L of 1X BlueJuice Loading Dye (Invitrogen™) was added to each tube, followed by loading of the solution onto a 10% TBE gel (20 μ L per well). The gel was run at 50V for 120 minutes at room temperature in x1 TBE buffer, upon completion the gel was stained with SYBR™ Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO, Invitrogen™) (5 μ L dissolved in 50 mL of milliQ water) for 30 minutes. The gel was visualised using the appropriate filter with ImageQuant LAS 4000.

6.2.5.2 4WJ promotion experiment

A 1.25 μ M solution of stoichiometric amounts of oligonucleotides X, R, H and B was prepared in Tris borate pH 8.0 buffer. A total of eight concentrations were tested for

each compound in 1 in 2 dilutions prepared in separate eppendorf tubes, each containing 50 µL of the DNA solution and 1 µL of compound (x50 of the final concentration). The tubes were incubated for 1 hour at room temperature. 1 µL of 1X BlueJuice Loading Dye (Invitrogen™) was added to each tube, followed by loading of the solution onto a 10% TBE gel (20 µL per well). The gel was run at 50V for 120 minutes at room temperature in x1 TBE buffer, upon completion the gel was stained with SYBR™ Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO, Invitrogen™) (5 µL dissolved in 50 mL of milliQ water) for 30 minutes. The gel was visualised using the appropriate filter with ImageQuant LAS 4000.

6.2.6 EMSA Three-Way DNA Junction Binding Assay

The oligonucleotides used in the 3WJ binding assay are:

S1 = 5' – CGGAACGGCACTCG - 3'

S2 = 5' – CGAGTGCAGCGTGG - 3'

S3 = 5' - CCACGCTCGTTCCG - 3'

A 1 µM solution of stoichiometric amounts of oligonucleotides S1, S2 and S3 was prepared in Tris borate pH 8.0 buffer. A total of eight concentrations were tested for each compound in 1 in 2 dilutions prepared in separate eppendorf tubes, each containing 50 µL of the DNA solution and 1 µL of compound (x50 of the final concentration). The tubes were incubated for 1 hour at room temperature. 1 µL of 1X BlueJuice Loading Dye (Invitrogen™) was added to each tube, followed by loading of the solution onto a 10% TBE gel (20 µL per well). The gel was run at 50V for 105 minutes at room temperature in x1 TBE buffer, upon completion the gel was stained with SYBR™ Gold Nucleic Acid Gel Stain (10,000X Concentrate in DMSO, Invitrogen™) (5 µL dissolved in 50 mL of milliQ water) for 30 minutes. The gel was visualised using the appropriate filter with ImageQuant LAS 4000.

6.2.7 FRET assay

The Junction 3 (J3) oligonucleotides used in this assay are:

B = 5' – CCTCCGTCCTAGCAAGGGGCTGCTACCGGAAGGG - 3'

H = 5' – CCCTTCCGGTAGCAGCCTGAGCGGTGGTTGAAGG - 3'

X = 5' – **TAMRA** – CCAGACTGCAGTTGAGTCCTTGCTAGGACGGAGG - 3'

R = 5' – **FAM** – CCTTCAACCACCGCTCAACTCAACTGCAGTCTGG - 3'

Stoichiometric amounts of oligonucleotides X, R, B and H were used to prepare a 50 nM solution of DNA in 20 mM Tris·HCl, 62.5 mM NaCl pH 7.5, the solution was heated to 90 °C and allowed to reach room temperature overnight. In a 96-well black low binding plate, 100 µL of the 50 nM solution were added, followed by 1 µL of compound in DMSO, a total of eight concentrations were tested in triplicate. Two controls were included, x3 no compound and x3 with the addition of 1 µL of DMSO. The plate was incubated for 1 hour at room temperature in the dark, and read on a ClarioStar Plate Reader (excitation 490 nm, emission 550 nm (FAM) and 600 nm (TAMRA)). The readings were reported as E_{FRET} equal to the FAM emission over TAMRA emission, plotted using GraphPad Prism version 8.0.

6.2.8 FRET-based melting assay

The labelled oligonucleotides used in this assay are:

3WJ: 5' – **FAM** - ACTCTTCTCGTTTTTTTCGAGAGCGACTTTTTTGTTCGCAGAGT – **TAMRA** - 3'

dsDNA: 5' – **FAM** - TATAGCTATATTTTTTTTATAGCTATA – **TAMRA** – 3'

4WJ:

S1: 5' – **FAM** – GGTGGCGAGAGCGACGATCCTTTTTTGGATCGTCGCAGAGTTG ACC – 3'

S2: 5' – GGTCAACTCTCTATGCTACCTTTTTTGGTAGCATAGTCTCGCCACC – **TAMRA** – 3'

A solution of labelled DNA was prepared as a 220 nM solution in 10 mM lithium cacodylate buffer (10 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl, pH 7.2) and heated to 90 °C, left to reach room temperature overnight. Samples were prepared in strip tubes (QIAgen) with a total volume of 20 µL, made up of 18 µL of DNA solution (final concentration 200 nM) and 2 µL of 100 µM of compound in DMSO (final concentration 10 µM). The fluorescence melting curves were measured in a QIAgen Roto-Gene Q-series PCR machine, initially holding the samples at 25 °C for 5 minutes, followed by heating of 1 °C increments, holding the temperature for 1 minute between each step, up to 95 °C. Each compound was tested in triplicate, whilst each run included controls, x3 controls of the DNA solution with the addition of 2 µL of buffer, to determine the DNA melting temperature in the absence of compound, and x3 controls with the addition of 2 µL of DMSO, to account for the DMSO effect on the

melting curve calculated. The emission of FAM was used to monitor melting, the excitation wavelength used was 470 nm and emission detection was measured at 510 nm. The DNA melting curves were determined using the first derivative of the melting curves, GraphPad Prism version 8.0 was used to plot the melting curve graphs.

6.2.9 FRET-based competition melting assay

The unlabelled oligonucleotides used in this assay are the same as the labelled sequences, presented in **section 5.2.7**, without any fluorescent tags.

A solution of labelled DNA was prepared as a 285 nM solution in 10 mM lithium cacodylate buffer (10 mM lithium cacodylate, 10 mM KCl, 90 mM LiCl, pH 7.2) and heated to 90 °C, left to reach room temperature overnight. Samples were prepared in strip tubes (QIAgen) with a total volume of 20 µL, made up of 16 µL of labelled DNA solution (final concentration 200 nM), 2 µL of unlabelled DNA competitor (final concentration of 200 nM for 1 equivalent experiments, 2 µM for 10 equivalents experiments and 10 µM for 50 equivalents experiments) and 2 µL of 100 µM of compound in DMSO (final concentration 10 µM). The fluorescence melting curves were measured in a QIAgen Roto-Gene Q-series PCR machine, initially holding the samples at 25 °C for 5 minutes, followed by heating of 1 °C increments, holding the temperature for 1 minute between each step, up to 95 °C. Each compound was tested in triplicate, whilst each run included controls, x3 controls of the DNA solution with the addition of 2 µL of buffer, to determine the DNA melting temperature in the absence of compound, and x3 controls with the addition of 2 µL of DMSO, to account for the DMSO effect on the melting curve calculated. The emission of FAM was used to monitor melting, the excitation wavelength used was 470 nm and emission detection was measured at 510 nm. The DNA melting curves were determined using the first derivative, GraphPad Prism version 8.0 was used to plot the melting curve graphs.

6.2.10 Fluorescence Titration

The unlabelled oligonucleotides used in this experiment are:

3WJ: 5' - ACTCTTCTCGTTTTTTTCGAGAGCGACTTTTTTGTGCGCAGAGT – 3'

dsDNA: 5' – TATAGCTATATTTTTTTTATAGCTATA – 3'

4WJ:

S1: 5' – GGTGGCGAGAGCGACGATCCTTTTTTGGATCGTCGCAGAGTTGACC –3'

S2: 5' – GGTCAACTCTCTATGCTACCTTTTTTGGTAGCATAGTCTCGCCACC – 3'

Solutions of 1 μM of compound in 400 μL of 10 mM Tris·HCl, pH 7.4 were prepared and 1 μL aliquots were added of annealed unlabelled DNA solutions (heated to 90 °C and allowed to reach room temperature overnight). A total of 13 additions were carried out (total of 13 μL volume increase), with final concentrations of DNA 0.1 μM , 0.2 μM , 0.3 μM , 0.4 μM , 0.5 μM , 0.75 μM , 1 μM , 1.5 μM , 2 μM , 3 μM , 4 μM and 5 μM . All the measurements were recorded on a Hitachi F-4500 fluorescence spectrometer, in right angle mode, at room temperature and using quartz cuvette with a 1 cm path length. The fraction of bound complex can be represented as the relative fluorescence (F/F_0) (with F_0 named as the fluorescence of the compound read in the absence of DNA), which is plotted against DNA concentration added. The binding curve was fitted to a one-site binding model using GraphPad Prism version 8.0.

6.3 Cell viability assays

6.3.1 Cell culture

MiaPaCa-2 cells were cultured in DMEM/F12 and HL-60 and A375 cells in RPMI-1640, both supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. HL-60 cells were subcultured 1 in 20 twice a week, and grown at 37 °C, 5% CO_2 .

A375 and MiaPaCa-2 cells were passaged every 3 – 4 days, at approximately 80% confluency, determined by white light microscopy. During the subculture process the media was removed from the culture flask, the cells were washed with prewarmed PBS (5 mL), after which TrypLE express (GIBCO) (2 mL) was added and the cells were incubated at 37 °C, 5% CO_2 for a few minutes. Once the cells lifted away from the flask, the TrypLE was deactivated by the addition of media (10 mL) to the flask, all but 1 mL of the cell suspension was removed from the flask. Fresh media (9 mL) was added to the flask, and the cells were incubated at 37 °C, 5% CO_2 .

6.3.2 MTS assay

Cell viability assessment by MTS assay was performed using CellTiter 96® Aqueous One Solution Cell Proliferation Assay Kit (Promega). HL-60 cells were seeded at a density of 3×10^4 cells in 100 μL , MiaPaCa-2 were seeded at 6×10^3 cells per 100 μL and A375 cells were seeded at 5×10^3 cells per 100 μL , in 96 well plates, and incubated overnight at 37 °C, 5% CO_2 . Cells were treated in triplicate (1% DMSO v/v per well) for 72 hours. Control wells were included on each plate, x3 well containing only media, x3 well containing untreated cells, x3 well containing cells treated with DMSO and x3 well containing cells treated with doxorubicin (10 μM) or gemcitabine (10 μM), used as cell death controls. Each well was treated with 10 μL MTS reagent (3 hours for HL-60 cells, 90 mins for MiaPaCa-2 cells, 120 mins for A375 cells). The absorbance was measured at 492 nm using the BMG Labtech POLARstar OPTIMA microplate reader. The background absorbance was corrected via subtraction of the media control wells and cell viability was calculated as a percentage of the growth controls (1% DMSO v/v). IC_{50} values were determined using GraphPad Prism Version 8.0, using the four-parameter logistic model.

6.3.3 Assessment of synergism by MTS

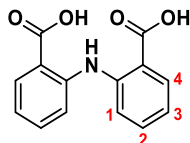
MiaPaCa-2 cells were seeded at a density of 6×10^3 cells per 100 μL in 96 well plates and incubated overnight. The media was removed and replaced by 50 μL of media containing compound (2-fold final concentration) and 50 μL of media containing a DNA repair inhibitor (2-fold final concentration). A total of six concentrations of compound against six concentrations of DNA repair inhibitor were assessed. Each plate was repeated in triplicate and incubated for 72 hours. Media was replaced and each well was treated with 10 μL MTS reagent for 90 minutes. The absorbance was measured at 492 nm using the BMG Labtech POLARstar OPTIMA microplate reader. The background absorbance was corrected via subtraction of the media control wells and cell viability was calculated as a percentage of the growth controls (1% DMSO v/v).

The results were analysed using a Loewe additivity 4-parameter model, combination index heat maps and isobologram plots were plotted for each combination.

6.4 Experimental procedures and characterisation

6.4.1 Chapter 2

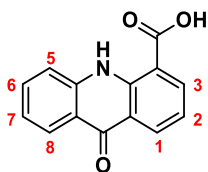
6.4.1.1 2,2'-iminodibenzoic acid (**2.61**)



2-bromobenzoic acid (3.015 g, 14.9 mmol, 1 eq.) and anthranilic acid (4.505 g, 16.40 mmol, 1.1 eq.) were stirred in DMF (20 mL) with potassium carbonate (6.007 g, 44 mmol, 3 eq.) and copper powder (0.200 g, 2.9 mmol, 0.2 eq.) under reflux overnight. On cooling, cold water was added (60 mL) and two spoonsful of activated charcoal to quench the reaction. The solution was purified through a celite plug, removing the copper powder and the charcoal. Concentrated hydrochloric acid was added dropwise to the filtrate, white precipitate starts to form. The white solid was collected via filtration and freeze dried to remove any traces of water (3.010 g, 78%). The data matched that reported in literature.³⁵²

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.90 (dd, *J* = 7.9, 1.5 Hz, 2H, H₄), 7.50 – 7.39 (m, 4H, H₁₊₂), 6.94 (ddd, *J* = 7.9, 6.4, 1.5 Hz, 2H, H₃). **¹³C NMR** (100 MHz, DMSO- *d*₆) δ 168.42 (COOH), 143.60, 133.35, 131.81, 119.99, 117.67, 117.58. **HRMS** (ES+) calculated for C₁₄H₁₁NO₃ [M+H]⁺ 258.0766 found 258.0776.

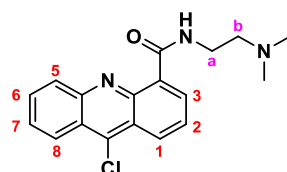
6.4.1.2 9-Oxo-9,10-dihydroacridine-4-carboxylic acid (**2.62**)



2,2'-Iminodibenzoic acid (2.506 g, 12 mmol) was dissolved in concentrated H₂SO₄ (25 mL) and the reaction was heated at 100 °C for one hour. On cooling the solution was poured over ice water (200 mL) to give a dark yellow precipitate. After washing well with water, the solid was dissolved in a 1:1 mixture of methanol (50 mL) and aqueous NaOH (2 M) (50 mL). The filtrate was placed in an ice bath then acidified with glacial acetic acid. The resulting precipitate was collected by filtration, washed with water and dried under vacuum yielding a bright yellow solid (1.731 g, 73%). The data matched that reported in literature.³⁵¹

¹H NMR (400 MHz, DMSO-*d*₆) δH ppm: 11.97 (s, 1H, NH), 8.50 (dd, *J* = 1.6, 8.0 Hz, 1H, H₁), 8.44 (dd, *J* = 1.8, 7.4 Hz, 1H, H₈), 8.24 (d, *J* = 7.6 Hz, 1H, H₃), 7.80-7.74 (m, 2H, H₅₊₆), 7.37-7.31 (m, 2H, H₂₊₇). **¹³C NMR** (100 MHz, DMSO- *d*₆) δ 176.64 (C=O), 169.21 (COOH), 141.26, 139.98, 136.98, 134.20, 132.49, 125.96, 122.40, 121.67, 120.65, 120.32, 118.66, 115.04. **HRMS** (ES+) calculated for C₁₄H₉NO₃ [M+H]⁺ 240.0661 found 240.0672.

6.4.1.3 9-Chloro-N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**)



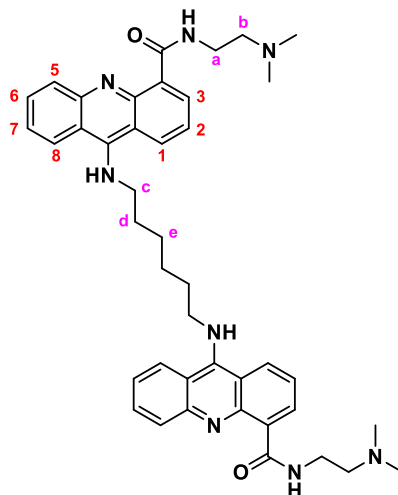
9-Oxo-9,10-dihydroacridine-4-carboxylic acid (**2.62**) (0.685 g, 2.86 mmol) was heated under reflux in thionyl chloride (10 mL) containing two drops of DMF for one hour. The solution was cooled and dried under vacuum, toluene was added (10 mL) and the solution was evaporated to remove all traces of thionyl chloride, this was repeated a total of three times, yielding the intermediate 9-chloroacridine-4-carbonyl chloride as a bright yellow solid.

N,N-dimethylethylenediamine (1.260 mL, 11.70 mmol) was added to anhydrous CH₂Cl₂ (20 mL) and the solution was cooled to 0 °C. The intermediate was cooled to -5 °C and the cold amine solution was added quickly in one aliquot, the reaction was stirred at 30 °C for forty five minutes under N₂. The reaction was washed with 10% Na₂CO₃ three times and once with brine, the organic layer was dried over MgSO₄, filtered and the solvent was removed under vacuum. The product was purified with column chromatography (10-20% MeOH in CH₂Cl₂), yielding a yellow oil that was triturated with hexane to afford a pale yellow solid (580 mg, 62%). The data matched that reported in literature.³⁵¹

¹H NMR (400 MHz, CDCl₃) δ 11.91 (s, 1H, NH), 8.97 (dd, *J* = 7.1, 1.5 Hz, 1H, H₁), 8.55 (dd, *J* = 8.7, 1.5 Hz, 1H, H₃), 8.42 – 8.35 (m, 1H, H₈), 8.20 (d, *J* = 8.7 Hz, 1H, H₅), 7.84 (ddd, *J* = 8.7, 6.6, 1.3 Hz, 1H, H₆), 7.77 – 7.61 (m, 2H, H₂₊₇), 3.78 (q, *J* = 6.1 Hz, 2H, H_a), 2.72 (t, *J* = 6.1 Hz, 2H, H_b), 2.45 (s, 6H, (CH₃)₂). **¹³C NMR** (100 MHz, CDCl₃) δ 165.40 (CONH), 147.38, 146.31, 142.76, 135.44, 131.38, 129.38, 128.95, 128.40, 127.44, 126.56, 124.63, 124.44, 123.77, 77.36, 77.04, 76.73, 58.16 (CH₂-

$N(CH_3)_2$, 45.44 (2CH₃), 37.92 (CONH-CH₂). **HRMS** (ES⁺) calculated for C₁₈H₁₈ClN₃O [M+H]⁺ 328.1137 found 328.1217.

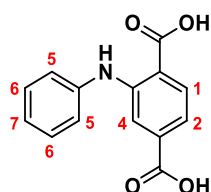
6.4.1.4 9,9'-(Hexane-1,6-diylbis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.18**)



9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (200 mg, 0.61 mmol, 2 eq.) and excess dry phenol (1.525 g) were heated at 110 °C and stirred for fifteen minutes. The solution was then cooled to 55 °C and 1,6-diaminohexane (35 mg, 0.31 mmol, 1 eq.) was added, the reaction stirred overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel. CH₂Cl₂ was used to transfer remaining material from round bottom flask to the separating funnel. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. The crude product was dried onto silica and column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright yellow oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (151 mg, 59%). The data matched that reported in literature.³¹⁷

R_f = 0.29 (50 % MeOH in EtOAc + 1% Et₃N). **IR** ν_{max} (neat) / cm⁻¹ 3362 (NH), 3231 (NH amide), 2940 (CH₂/CH₃), 1619 (C=O). **¹H NMR** (400 MHz, MeOH-*d*₄) δ 8.70 (dd, *J* = 8.6, 1.1 Hz, 2H, H₁), 8.57 (dd, *J* = 7.5, 1.1 Hz, 2H, H₃), 8.51 (d, *J* = 8.8 Hz, 2H, H₈), 7.89 - 8.02 (m, 4H, H₅₊₆), 7.64 - 7.55 (m, 4H, H₂₊₇), 4.15 (t, *J* = 7.3 Hz, 4H, H_c), 3.91 (t, *J* = 5.9 Hz, 4H, H_a), 3.49 (t, *J* = 5.9 Hz, 4H, H_b), 3.02 (s, 12H, (CH₃)₄), 1.99 - 2.08 (m, 4H, H_d), 1.62 - 1.56 (m, 4H, H_e). **HRMS** (ES⁺) calculated for C₄₂H₅₀N₈O₂ [M+H]⁺ 699.4057 found 699.4135. **UV** λ_{max} = 416 nm. **HPLC**: *t_R* = 16.1 min.

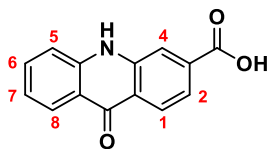
6.4.1.5 2-(Phenylamino)terephthalic acid (**2.66**)



Potassium carbonate (6.402 g, 46 mmol) was dissolved in distilled water (150 mL), followed by the addition of 2-bromoterephthalic acid (7.506 g, 31 mmol), aniline (5.6 mL, 61 mmol), pyridine (451 μ L), copper powder (150 mg) and copper iodide (150 mg). The solution was stirred under reflux for four hours. On cooling 5% NaOH was added to dissolve any product, followed by filtration through a celite-silica-celite layered sintered filter. The residue was washed with NaHCO₃ to remove any remaining copper from the catalysts. The collected filtrate was acidified dropwise with concentrated HCl, forming a yellow precipitate. The solution was filtered and the product was isolated as a bright yellow solid (7.627 g, 97%). The data matched that reported in literature.³⁵³

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.61 (s, 1H, NH), 7.98 (d, *J* = 8.2 Hz, 1H, H₁), 7.74 (d, *J* = 1.4 Hz, 1H, H₄), 7.45 - 7.36 (m, 2H, H₆), 7.33 - 7.23 (m, 3H, H₂₊₅), 7.14 (tt, *J* = 7.3, 1.2 Hz, 1H, H₇). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ ppm: 171.48 (COOH), 168.86 (COOH), 147.55, 140.49, 135.92, 132.66, 130.12 (2C), 124.30, 122.61 (2C), 117.86, 116.03, 114.75. **HRMS** (ES⁺) calculated for C₁₄H₁₁NO₄ [M+H]⁺ 258.0766 found 258.0766.

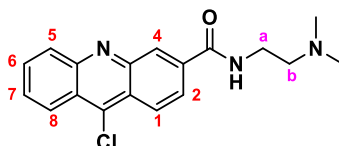
6.4.1.6 9-Oxo-9,10-dihydroacridine-3-carboxylic acid (**2.67**)



2-(Phenylamino)terephthalic acid (**2.66**) (1.082 g, 4.19 mmol) was dissolved in polyphosphoric acid (15 mL), heated to 120 °C and stirred for three hours. The reaction mixture was poured into hot water, forming a green precipitate, which was collected via filtration and washed well with hot water. The collected material was dissolved in a 1:1 mixture of methanol (40 mL) and 2M NaOH (40 mL), placed in ice and acidified with glacial acetic acid. The formed precipitate was filtered, washed with water and dried under vacuum, to yield a green-yellow solid (740 mg, 74%). The data matched that reported in literature.³⁵¹

¹H NMR (400 MHz, DMSO-*d*₆) δ 11.94 (s, 1H, NH), 8.30 (d, *J* = 8.4 Hz, 1H, H₁), 8.24 (d, *J* = 8.1 Hz, 1H, H₈), 8.18 (s, 1H, H₄), 7.81 – 7.69 (m, 2H, H₂₊₆), 7.55 (d, *J* = 8.4 Hz, 1H, H₅), 7.29 (t, *J* = 8.1 Hz, 1H, H₇). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ 176.58 (C=O ring), 166.76 (COOH), 141.31, 140.39, 134.73, 133.88, 126.50, 126.06, 123.05, 121.77, 120.77, 120.66, 119.11, 117.53. **HRMS** (ES⁺) calculated for C₁₄H₉NO₃ [M+H]⁺ 240.0582 found 240.0610.

6.4.1.7 9-Chloro-N-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**)



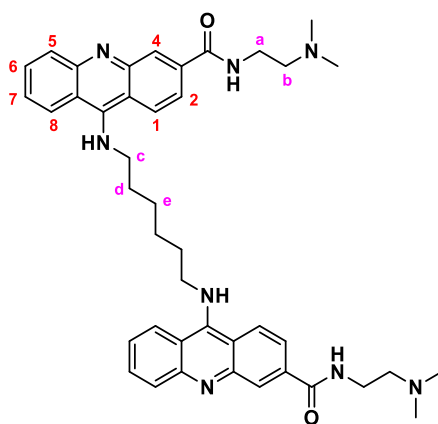
9-Oxo-9,10-dihydroacridine-3-carboxylic acid (**2.67**) (903 mg, 3.76 mmol) was heated under reflux in thionyl chloride (10 mL) containing two drops of DMF for one hour. The solution was cooled and dried under vacuum, toluene was added and the solution was evaporated to remove all traces of thionyl chloride, this was repeated a total of three times, to afford the intermediate 9-chloroacridine-4-carbonyl chloride as a bright yellow solid.

N,N-dimethylethylenediamine (1.650 g, 15.04 mmol) was added to anhydrous CH₂Cl₂ and the solution was cooled to 0 °C. The intermediate was cooled to -5 °C and the cold amine solution was added quickly in one aliquot, the reaction was stirred at 30 °C for forty five minutes under N₂. The reaction was washed with 10% Na₂CO₃ three

times and once with brine, the organic layer was dried over MgSO_4 , filtered and the solvent was removed under vacuum. The product was purified with column chromatography (10-20% MeOH in CH_2Cl_2), yielding a yellow oil that was triturated with hexane to afford a pale yellow solid (739 mg, 60%). The data matched that reported in literature.³⁵¹

^1H NMR (400 MHz, CDCl_3) δ 8.55 (dd, $J = 1.7, 0.7$ Hz, 1H, H_4), 8.53 – 8.40 (m, 2H, H_{1+8}), 8.22 (dt, $J = 8.8, 1.0$ Hz, 1H, H_5), 8.13 (dd, $J = 9.1, 1.7$ Hz, 1H, H_2), 7.84 (ddd, $J = 8.8, 6.6, 1.4$ Hz, 1H, H_6), 7.68 (ddd, $J = 8.8, 6.6, 1.0$ Hz, 1H, H_7), 3.65 – 3.57 (m, 2H, H_a), 2.61 – 2.54 (m, 2H, H_b), 2.30 (s, 6H, $(\text{CH}_3)_2$). **^{13}C NMR** (100 MHz, CDCl_3) δ ppm: 166.65 (C=O), 149.66, 148.34, 141.42, 136.61, 131.19, 129.99, 128.17, 127.81, 125.70, 125.49, 125.34, 125.02, 124.88, 57.60 ($\text{CH}_2\text{-N}(\text{CH}_3)_2$), 45.27 ($(\text{CH}_3)_2$), 37.51 (NH- CH_2). **HRMS** (ES+) calculated for $\text{C}_{18}\text{H}_{18}\text{ClN}_3\text{O}$ $[\text{M}+\text{H}]^+$ 328.1217 found 328.1227.

6.4.1.8 9,9'-(Hexane-1,6-diylbis(azanediy))bis(*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.75**)

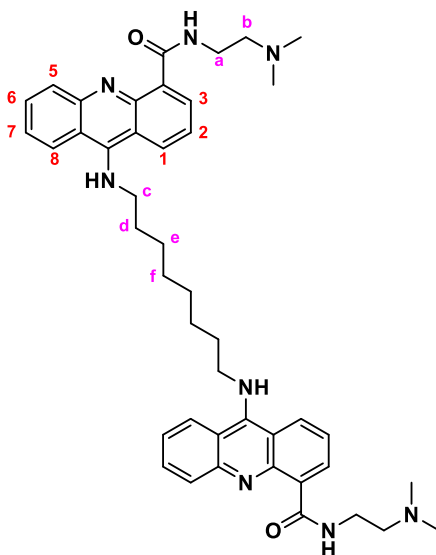


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (40 mg, 0.12 mmol) and excess dry phenol (1.004 g) were heated at 110 °C and stirred for fifteen minutes. The solution was then cooled to 55 °C and 1,6-diaminohexane (7 mg, 0.06 mmol) was added, the reaction stirred overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et_3N). On cooling to room temperature, 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel. CH_2Cl_2 was used to transfer remaining material from the round bottom flask to the separating funnel. The organic layer was separated, and the aqueous layer was extracted successively with CH_2Cl_2 (3 x 10 mL). The organic layers were combined, dried over MgSO_4 and filtered, the solvent was removed under vacuum to yield an orange oil.

The crude product was dried onto silica and column chromatography (0 - 50 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright yellow oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (19 mg, 38%). The data matched that reported in literature.³¹⁶

IR ν_{max} (neat) / cm⁻¹ 3328 (NH), 3119 (CH), 2937 (CH₂/CH₃), 1633 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.62 (d, *J* = 9.0 Hz, 2H, H₁), 8.53 (d, *J* = 8.6 Hz, 2H, H₈), 8.37 (dd, *J* = 5.7, 1.7 Hz, 2H, H₄), 8.07 – 7.95 (m, 4H, H₂₊₆), 7.87 (d, *J* = 8.6 Hz, 2H, H₅), 7.58 (m, 2H, H₇), 4.21 (m, 4H, H_c), 3.90 (t, *J* = 5.9 Hz, 4H, H_a), 3.55 – 3.44 (t, *J* = 5.9 Hz, 4H, H_b), 3.05 (s, 12H, (CH₃)₄), 2.08 (br s, 4H, H_d), 1.65 (br s, 4H, H_e). **LRMS** calculated for C₄₂H₅₀N₈O₂ (M+H)⁺ 699.4 found 699.3. **UV** λ_{max} = 421 nm. **HPLC**: *t*_R = 12.2 min.

6.4.1.9 9,9'-(Octane-1,8-diylbis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.76**)

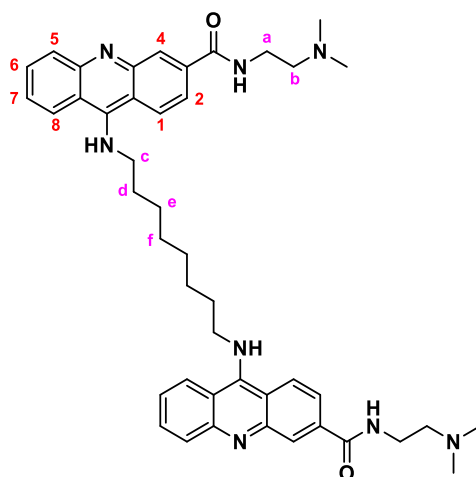


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (150 mg, 0.45 mmol, 2 eq.) and excess dry phenol (1.005 g) were heated at 110 °C and stirred for fifteen minutes. The solution was then cooled to 55 °C and 1,8-diaminooctane (33 mg, 0.23 mmol, 1 eq.) was added, the reaction stirred overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a

separating funnel. CH₂Cl₂ was used to transfer remaining material from round bottom flask to the separating funnel. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright yellow oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected via filtration, the bright yellow compound was freeze dried overnight, removing any traces of water (137 mg, 70%). The data matched that reported in literature.³¹⁶

IR ν_{max} (neat) / cm⁻¹ 3365 (NH), 3224 (NH amide), 2931 (CH₂/CH₃), 1620 (C=O). **¹H NMR** (400 MHz, MeOH-*d*₄) δ 8.72 (d, *J* = 8.4 Hz, 2H, H₁), 8.57 (d, *J* = 7.5 Hz, 2H, H₃), 8.52 (d, *J* = 8.4 Hz, 2H, H₈), 8.02-7.98 (m, 2H, H₆), 7.91 (d, *J* = 8.8 Hz, 2H, H₅), 7.65-7.58 (m, 4H, H₂₊₇), 4.14 (t, *J* = 7.4 Hz, 4H, H_c), 3.91 (t, *J* = 5.6 Hz, 4H, H_a), 3.52 (t, *J* = 5.6 Hz, 4H, H_b), 3.05 (s, 12H, (CH₃)₄), 2.02-1.96 (m, 4H, H_d), 1.51-1.46 (m, 8H H_{e+f}). **HRMS** (ES⁺) calculated for C₄₄H₅₄N₈O₂ [M+H]⁺ 727.4448 found 727.4370. **UV** λ_{max} = 420 nm. **HPLC**: *t_R* = 18.8 min.

6.4.1.10 9,9'-(Octane-1,8-diylbis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.77**)

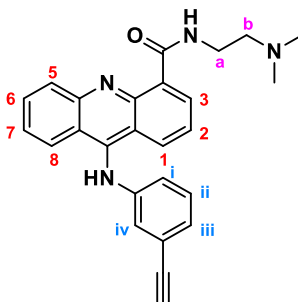


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (70 mg, 0.21 mmol) and excess dry phenol (1.503 g) were heated at 110 °C and stirred for fifteen minutes. The solution was cooled to 55 °C and 1,8-diaminooctane (18 mg, 0.11 mmol) was added, the reaction stirred overnight. TLC confirmed the formation of the product

(50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel. CH₂Cl₂ was used to transfer remaining material from round bottom flask to the separating funnel. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright yellow oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (55 mg, 60%).

IR ν_{max} (neat) / cm⁻¹ 3278 (NH), 3025 (CH), 2934 (CH₂/CH₃), 1634 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.62 (d, *J* = 8.9 Hz, 2H, H₁), 8.52 (d, *J* = 8.6 Hz, 2H, H₈), 8.35 (d, *J* = 1.7 Hz, 2H, H₄), 7.99 (m, 4H, H₂₊₆), 7.89 – 7.83 (m, 2H, H₅), 7.59 (m, 2H, H₇), 4.17 (t, *J* = 7.4 Hz, 4H, H_c), 3.87 (t, *J* = 5.9 Hz, 4H, H_a), 3.48 (t, *J* = 5.9 Hz, 4H, H_b), 3.03 (s, 12H, (CH₃)₄), 2.00 (d, *J* = 7.4 Hz, 4H, H_d), 1.48 (m, 8H, H_{e+f}). **LRMS** calculated for C₄₄H₅₄N₈O₂ [M+H]⁺ 727.4 found 727.4. **UV** λ_{max} = 423 nm. **HPLC**: *t*_R = 12.2 min.

6.4.1.11 *N*-(2-(Dimethylamino)ethyl)-9-(3-ethylphenylamino)acridine-4-carboxamide (2.85)

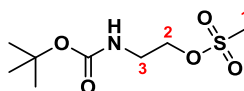


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (300 mg, 0.92 mmol) and excess dry phenol (1.503 g) were stirred at 110 °C for fifteen minutes. On cooling to 55 °C 3-ethynylaniline (96 μ L, 0.92 mmol) was added and the reaction was stirred for 5 hours at 55 °C. On cooling to room temperature 2M NaOH was added (20 mL) was added to the reaction. The solution was extracted with CH₂Cl₂. The organic phase was separated and the aqueous phase was extracted with CH₂Cl₂ (3 x 10 mL). The organic layers were combined and dried over MgSO₄ and filtered. Column

chromatography (0 - 3% MeOH in EtOAc + 1% Et₃N) yielded the desired product as an orange oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (5 mL) and stirred for 10 minutes, followed by precipitation with EtOAc and the product was collected by filtration. The bright yellow compound was freeze dried overnight (308 mg, 82%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm⁻¹ 3268 (NH/CH), 2961 (CH₂/CH₃), 1616 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.62 (s, 1H, H₁), 8.39 (d, *J* = 7.6 Hz, 1H, H₃), 8.08 (m, 2H, H₈₊₅), 7.91 (t, *J* = 7.7 Hz, 1H, H₆), 7.56 – 7.09 (m, 6H, H_{2+7+i+ii+iii+iv}), 3.98 (t, *J* = 7.3 Hz, 2H, H_a), 3.63 (q, *J* = 7.3 Hz, 1H, H_b), 3.54 (m, 2H, H_b+CH alkyne), 3.06 (s, 6H, (CH₃)₂). **LRMS** calculated for C₄₄H₅₄N₈O₂ (M+H)⁺ 409.1 found 409.0.

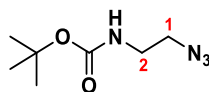
6.4.1.12 2-Boc ethyl methane sulfonate (**2.89**)



Boc-ethanolamine (1.003 g, 6.2 mmol) was dissolved in small amount of dry CH₂Cl₂ and triethylamine (2.6 mL, 18.6 mmol) was added and the reaction was cooled to 0 °C. Mesyl chloride (480 μ L, 6.2 mmol) was added dropwise at 0 °C, the reaction was then allowed to warm up to room temperature and then stirred overnight under nitrogen. The reaction was diluted with water (20 mL) and poured into a separating funnel. The aqueous phase was extracted with CH₂Cl₂ (3 x 10 mL), the organic layers were combined and dried over MgSO₄ and filtered. The remaining solvent was evaporated off under vacuum yielding a yellow oil (1.261 g, 85%). The data matched that reported in literature.³²⁰

¹H NMR (400 MHz, CDCl₃) δ 4.89 (s, 1H, NH), 4.22 (t, *J* = 5.2 Hz, 2H, H₂), 3.40 (t, *J* = 5.2 Hz, 1H, H₃), 2.97 (s, 3H, H₁), 1.38 (s, 9H, (CH₃)₃). **¹³C NMR** (100 MHz, CDCl₃) δ 155.60 (C=O), 79.03 (C(CH₃)₃), 68.90 (CH₂), 40.51 (CH₂), 37.05 (CH₃), 25.15 (C(CH₃)₃)

6.4.1.13 2-Boc azidoethylcarbamate (**2.90**)

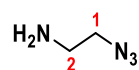


2-(*Tert*-butoxycarbonylamino)ethyl methane sulfonate (1.252 g, 5.22 mmol) was dissolved in dry DMF (10 mL), sodium azide (850 mg, 13.07 mmol) was added to the

solution and the reaction was heated to 67 °C and stirred for 1 hour. On cooling, water (20 mL) was added to the reaction and poured into a separating funnel and extracted aqueous phase with diethyl ether (3 x 10 mL) and washed combined organic phases with water (3 x 10 mL). The organic layers were dried over MgSO₄, filtered and evaporated remaining solvent off under vacuum yielding a lightly tinted yellow oil (690 mg, 71%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm⁻¹ 2100 (N₃). **¹H NMR** (400 MHz, CDCl₃) δ 4.84 (s, 1H, NH), 3.41 (t, J = 5.6 Hz, 2H, H₁), 3.29 (m, J = 5.6 Hz, 2H, H₂), 1.44 (s, 9H, (CH₃)₃). **¹³C NMR** (100 MHz, CDCl₃) δ ppm: 167.45 (C=O), 80.02 (C(CH₃)₃), 51.45 (CH₂), 40.32 (CH₂), 28.65 (C(CH₃)₃).

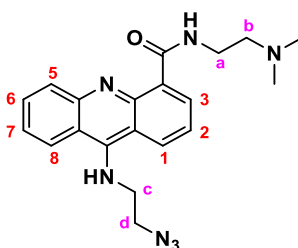
6.4.1.14 2-azidoethanamine hydrochloride (2.91)



Tert-Butyl 2-azidoethylcarbamate (700 mg, 3.75 mmol) was stirred in a mixture of 1 M HCl/EtOAc (10 mL) overnight at room temperature. The solvent was removed under vacuum to yield an oily yellow solid (307 mg, 95%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm⁻¹ 2100 (N₃). **¹H NMR** (400 MHz, D₂O) δ 3.74 (t, J = 5.6 Hz, 2H, H₁), 3.20 (t, J = 5.6 Hz, 2H, H₂). **¹³C NMR** δ ppm: 48.15 (CH₂), 38.80 (CH₂).

6.4.1.15 9-(2-Azidoethylamino)-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (2.86)

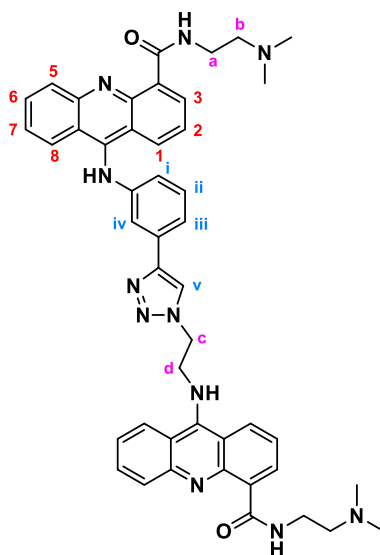


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (315 mg, 0.96 mmol) was stirred in excess phenol (1.012 g), heated to 100 °C and stirred for fifteen minutes. The reaction was cooled to 55 °C and 2-azidoethanamine hydrochloride (0.100 g, 0.81 mmol) was added to the reaction which was allowed to stir overnight at 55 °C. On cooling 2 M NaOH (20 mL) was added to the reaction and it was poured into a separating funnel, the organic layer was removed and the aqueous phase was extracted with CH₂Cl₂ (3 x 10 mL). The organic layers were combined and dried over

MgSO₄, filtered and the solvent was removed under vacuum. Column chromatography (0-20% MeOH in EtOAc + 1% Et₃N) yielded a bright yellow oil which was freeze dried overnight (311 mg, 86%). The data matched that reported in literature.³²⁰

IR v_{max} (neat) / cm⁻¹ 3305 (NH), 2940 (CH₂/CH₃), 2819 (CH₂/CH₃), 2098 (N₃), 1638 (C=O) **¹H NMR** (400 MHz, MeOH-*d*₄) δ 8.54 (d, *J* = 7.1 Hz, 1H, H₁), 8.36 (dd, *J* = 8.7, 1.5 Hz, 1H, H₃), 8.21 (dd, *J* = 8.9, 1.3 Hz, 1H, H₈), 7.86 (d, *J* = 8.5 Hz, 1H, H₅), 7.68 – 7.63 (m, 1H, H₆), 7.36 – 7.32 (m, 2H, H₂₊₇), 3.93 (t, *J* = 6.3, 2H, H_d), 3.70 (t, *J* = 6.5 Hz, 2H, H_a), 3.61 (t, *J* = 5.7 Hz, 2H, H_c), 2.73 (t, *J* = 6.5 Hz, 2H, H_b), 2.41 (s, 6H, (CH₃)₂). **LRMS** calculated for C₂₀H₂₃N₇O [M+H]⁺ 378.2 found 378.1.

6.4.1.16 *N*-(2-(Dimethylamino)ethyl)-9-(3-(1-(2-(4-(2-(dimethylamino)ethylcarbamoyl)acridin-9-ylamino)ethyl)-1H-1,2,3-triazol-4-yl)phenylamino)acridine-4-carboxamide (**2.87**)

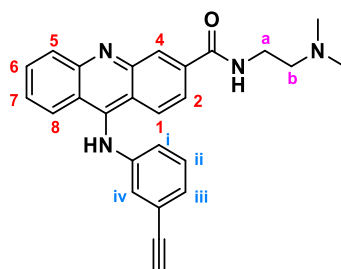


9-(2-Azidoethylamino)-*N*-(2-dimethylamino)ethyl)acridine-4-carboxamide (**2.86**) (100 mg, 0.27 mmol) and *N*-(2-(dimethylamino)ethyl)-9-(3-ethynylphenylamino)acridine-4-carboxamide (**2.85**) (108 mg, 0.27 mmol) were stirred in a 1:1 *t*-BuOH:H₂O mixture (1.5 mL) for two minutes. Sodium ascorbate (10 mol%, 26.5 μL of a 1 M stock) and copper sulphate (1 mol%, 2.65 μL of a 1 M stock) were added, the reaction was left to stir at room temperature for 72 hours. Once completion was confirmed by TLC, the solvents were removed *in vacuo*. The product was purified by column chromatography (10% - 50% MeOH in EtOAc + 1% Et₃N) to yield an orange oil. The compound was converted into the tetrahydrochloride salt by stirring in 1 M HCl in MeOH for ten minutes, followed by precipitation with EtOAc. The product was

collected via filtration, yielding a dark orange hygroscopic solid (160 mg, 64%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm^{-1} 3361 (NH), 3055 (CH), 2690 (CH_2/CH_3), 1619 (C=O). **^1H NMR** (400 MHz, $\text{MeOD-}d_4$) δ 8.41 (d, J = 7.3 Hz, 2H), 8.20 (m, 1H), 8.09 (dd, J = 8.6, 1.4 Hz, 1H), 7.95 (d, J = 8.6 Hz, 2H), 7.82 (s, 1H, triazole CH), 7.69 (d, J = 8.6 Hz, 1H), 7.59 (m, 1H), 7.53 – 7.45 (m, 1H), 7.34 – 7.09 (m, 6H), 7.05 (t, J = 1.8 Hz, 1H), 6.77 (dd, J = 8.5, 1.8 Hz, 1H), 4.61 (t, J = 5.6 Hz, 2H), 4.21 (t, J = 5.6 Hz, 2H), 3.67 (t, J = 6.5 Hz, 2H), 3.60 (t, J = 6.5 Hz, 2H), 2.70 (t, J = 6.5 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H), 2.39 (s, 6H, $(\text{CH}_3)_2$), 2.33 (s, 6H, $(\text{CH}_3)_2$). **HRMS** (ES+) calculated for $\text{C}_{46}\text{H}_{47}\text{N}_{11}\text{O}_2$ $[\text{M}+\text{H}]^+$ 786.3914 found 786.3992. **UV** λ_{max} = 417 nm. **HPLC**: t_{R} = 17.2 min.

6.4.1.17 *N*-(2-(Dimethylamino)ethyl)-9-(3-ethynylphenylamino)acridine-3-carboxamide (**2.93**)

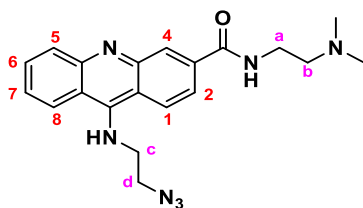


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (80 mg, 0.24 mmol) and excess dry phenol (1.010 g) were stirred at 110 °C for fifteen minutes. On cooling to 55 °C 3-ethynylaniline (36 μL , 0.31 mmol) was added and the reaction was stirred for 5 hours at 55 °C. On cooling to room temperature 2M NaOH was added (20 mL) to the reaction. The solution was extracted with CH_2Cl_2 . The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2 (3 x 10 mL). The organic layers were combined, dried over MgSO_4 and filtered. Column chromatography (10 - 50% MeOH in EtOAc + 1% Et_3N) yielded the desired product as an orange oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (5 mL), stirred for ten minutes, followed by precipitation with EtOAc and the product was collected by filtration. The bright yellow compound was freeze dried overnight (72 mg, 73%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm^{-1} 3232 (NH/CH alkyne), 2922 (CH_2/CH_3), 1634 (C=O). **^1H NMR** (400 MHz, $\text{MeOD-}d_4$) δ 8.50 (dd, J = 1.7, 0.6 Hz, 1H, H_4), 8.29 – 8.21 (m, 2H, H_{1+8}), 8.08 – 7.97 (m, 2H, H_{5+6}), 7.86 (dd, J = 9.1, 1.7 Hz, 1H, H_2), 7.61 – 7.43 (m, 5H,

H_{7+ii+iii+iv}), 3.87 (t, *J* = 5.9 Hz, 2H, H_a), 3.67 (s, 1H, C-H alkyne), 3.47 (t, *J* = 5.9 Hz, 2H, H_b), 3.02 (s, 6H, (CH₃)₂). **HRMS** (ES⁺) calculated for C₂₆H₂₄N₄O [M+H]⁺ 409.1950 found 409.2032.

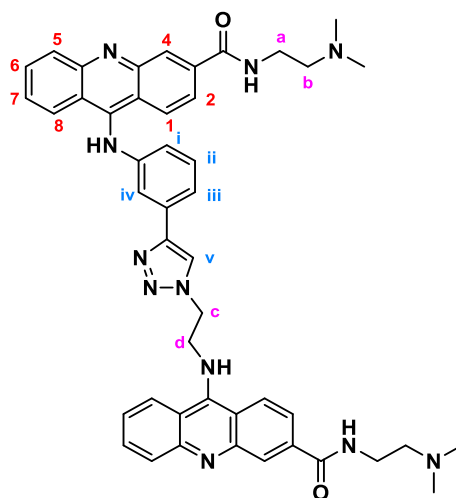
6.4.1.18 9-(2-Azidoethylamino)-N-(2-(dimethylamino)ethyl)acridine-3-carboxamide (2.92)



9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (90 mg, 0.27 mmol) was stirred in excess phenol (1.203 g) at 100 °C and stirred for fifteen minutes. The reaction was cooled to 55 °C and 2-azidoethanamine hydrochloride (32 mg, 0.37 mmol) was added to the reaction which was allowed to stir overnight at 55 °C. On cooling 2 M NaOH (20 mL) was added to the reaction and it was poured into a separating funnel, the organic layer was removed and the aqueous phase was extracted with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄, filtered and the solvent was removed under vacuum. Column chromatography (0 - 50% MeOH in EtOAc + 1% Et₃N) yielded a bright yellow oil which was freeze dried overnight (311 mg, 86%). The data matched that reported in literature.³²⁰

IR v_{max} (neat) / cm⁻¹ 3262 (NH), 2943 (CH₂/CH₃), 2826 (CH₂/CH₃), 2098 (N₃), 1642 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.47 (d, *J* = 9.0 Hz, 1H, H₈), 8.40 (d, *J* = 8.7 Hz, 1H, H₁), 8.28 (s, 1H, H₄), 7.90 – 7.75 (m, 3H, H₂₊₅₊₆), 7.47 (t, *J* = 9.0 Hz, 1H, H₇), 4.17 (t, *J* = 5.7 Hz, 2H, H_d), 3.75 (t, *J* = 5.7 Hz, 2H, H_c), 3.64 (t, *J* = 6.7 Hz, 2H, H_a), 2.76 (t, *J* = 6.7 Hz, 2H, H_b), 2.45 (s, 6H, (CH₃)₂). **HRMS** (ES⁺) calculated for C₂₀H₂₃N₇O [M+H]⁺ 378.1964 found 378.2049.

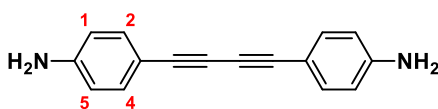
6.4.1.19 *N*-(2-(Dimethylamino)ethyl)-9-(3-(1-(2-(3-(2-(dimethylamino)ethylcarbamoyl)acridin-9-ylamino)ethyl)-1*H*-1,2,3-triazol-4-yl)phenylamino)acridine-3-carboxamide (**2.94**)



9-(2-Azidoethylamino)-*N*-(2-dimethylamino)ethyl)acridine-3-carboxamide (**2.92**) (37 mg, 0.09 mmol) and *N*-(2-(dimethylamino)ethyl)-9-(3-ethynylphenylamino)acridine-4-carboxamide (**2.93**) (40 mg, 0.09 mmol) were stirred in a 1:1 *t*-BuOH:H₂O mixture (1.2 mL) for two minutes. Sodium ascorbate (10 mol%, 11 μ L of a 1 M stock) and copper sulphate (1 mol%, 1 μ L of a 1 M stock) were added, the reaction was left to stir at room temperature for 72 hours. Once completion was confirmed by TLC, the solvents were removed *in vacuo*. The product was purified by column chromatography (20% - 50% MeOH in EtOAc + 1% Et₃N) to yield an orange oil. The compound was converted into the tetrahydrochloride salt by stirring in 1 M HCl in MeOH for ten minutes, followed by precipitation with EtOAc. The product was collected via filtration, yielding a dark orange hygroscopic solid (58.5 mg, 70%). The data matched that reported in literature.³²⁰

IR ν_{max} (neat) / cm⁻¹ 3300 (NH), 3063 (CH), 2691 (CH₂/CH₃), 1634 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.58 (d, *J* = 9.0 Hz, 1H), 8.52 – 8.45 (m, 2H), 8.41 (s, 1H, C-H triazole), 8.37 – 8.26 (m, 3H), 8.07 – 7.93 (m, 4H), 7.90 – 7.79 (m, 3H), 7.73 (d, *J* = 7.9 Hz, 1H), 7.64 – 7.45 (m, 3H), 7.41 (d, *J* = 7.9 Hz, 1H), 5.02 (t, *J* = 5.4 Hz, 2H, H_c), 4.85 – 4.76 (m, 2H, H_d), 3.90 – 3.80 (m, 4H, H_a), 3.44 – 3.49 (m, 4H, H_b), 3.02 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₄₆H₄₇N₁₁O₂ [M+H]⁺ 786.3914 found 786.3992. **UV** λ_{max} = 425 nm. **HPLC**: *t*_R = 16.0 min.

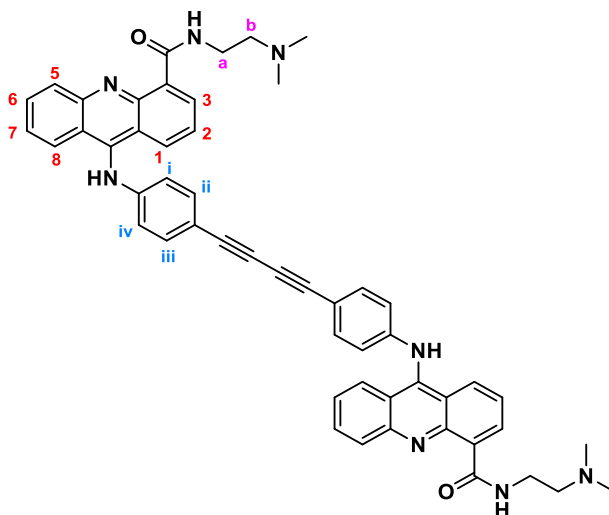
6.4.1.20 4,4'-(Buta-1,3-diyne-1,4-diyl)dianiline (**2.99**)



4-ethynylaniline (230 mg, 1.96 mmol) was dissolved in a 1:1 solution of MeOH and pyridine (v/v, 20 mL). Cu(OAc)₂ (910 mg, 5 mmol) was added and the solution was stirred under reflux for 2 hours. Upon completion, the solvents were evaporated, the remaining semi solid product was dissolved in water and extracted with EtOAc (3 x 10 mL), dried over MgSO₄, filtered and dried onto silica. Column chromatography (0 – 2% MeOH in CH₂Cl₂) yielded pure product as an orange solid (203 mg, 89%). The data matched that reported in literature.³⁵⁶

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.23 – 7.15 (m, 4H, H₁₊₅), 6.56 – 6.48 (m, 4H, H₂₊₄), 5.69 (s, 4H, (NH₂)₂). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ 150.17 (2C, NH₂-C), 133.54 (4C, C₁₊₅), 113.59 (4C, C₂₊₄), 106.42 (2C, ArC-C_{alkyne}), 82.91 (2C, C_{alkyne}), 72.03 (2C, C_{alkyne}). **HRMS** (ES⁺) calculated for C₁₆H₁₂N₂ [M+H]⁺ 233.1079 found 233.1082.

6.4.1.21 9,9'-(buta-1,3-diyne-1,4-diylbis(4,1-phenylene))bis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.100**)

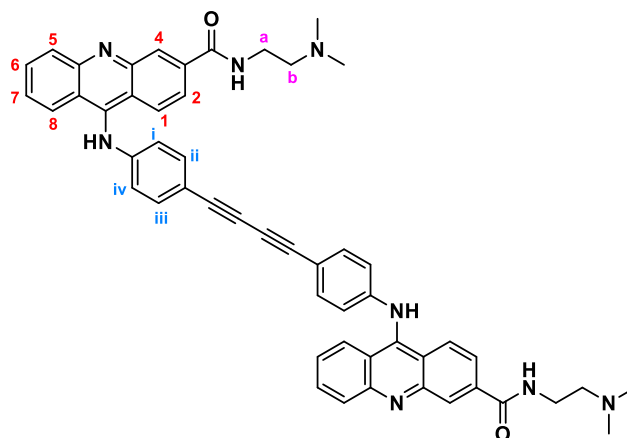


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (100 mg, 0.30 mmol) and excess dry phenol (1.504 g) were stirred at 110 °C for fifteen minutes. The solution was cooled to 55 °C and **2.99** (35 mg, 0.15 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added

to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (86 mg, 60%).

IR v_{max} (neat) / cm⁻¹ 3168 (NH), 3054 (CH), 2947 (CH₂/CH₃), 2765 (CH₂/CH₃), 1651 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.65 (d, *J* = 7.3 Hz, 2H), 8.52 (d, *J* = 8.6 Hz, 2H), 8.25 (d, *J* = 8.8 Hz, 2H), 8.17 – 8.04 (m, 4H), 7.69 (d, *J* = 8.6 Hz, 4H), 7.70 – 7.52 (m, 4H), 7.46 (d, *J* = 8.8 Hz, 4H), 3.95 (t, *J* = 5.7 Hz, 4H), 3.54 (t, *J* = 5.7 Hz, 4H), 3.06 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₅₂H₄₆N₈O₂ [M+H]⁺ 815.3822 found 815.3810. **UV** λ_{max} = 384 nm. **HPLC**: *t*_R = 20.3 min.

6.4.1.22 9,9'-(buta-1,3-diyne-1,4-diylbis(4,1-phenylene))bis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.101**)

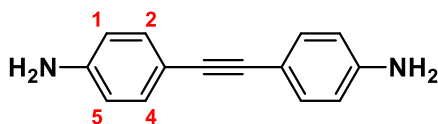


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (60 mg, 0.18 mmol) and excess dry phenol (1.009 g) were heated at 110 °C and stirred for fifteen minutes. The solution was cooled to 55 °C and **2.99** (21 mg, 0.09 mmol) mmol was added, the reaction stirred overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with

CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc, collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (41 mg, 48%).

IR ν_{max} (neat) / cm⁻¹ 3329 (NH), 2943 (CH₂/CH₃), 1599 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.65 (dd, *J* = 2.3 Hz, 2H), 8.23 (dt, *J* = 8.9, 0.9 Hz, 2H), 8.18 – 8.06 (m, 4H), 7.94 – 7.82 (m, 4H), 7.57 (ddd, *J* = 8.9, 6.6, 1.1 Hz, 2H), 7.35 – 7.25 (m, 4H), 7.12 – 7.05 (m, 2H), 6.90 – 6.83 (m, 4H), 3.62 (t, *J* = 6.8 Hz, 4H, H_a), 2.66 (t, *J* = 6.8 Hz, 4H, H_b), 2.36 (s, 12H, ((CH₃)₄)). **HRMS** (ES⁺) calculated for C₅₂H₄₆N₈O₂ [M+H]⁺ 815.3822 found 815.3802. **UV** λ_{max} = 415 nm. **HPLC**: *t*_R = 17.0 min.

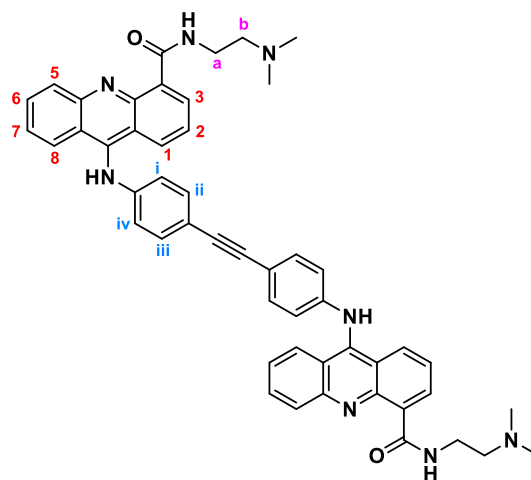
6.4.1.23 4,4'-(Ethyne-1,2-diyl)dianiline (2.104)



4-iodaniline (550 mg, 2.51 mmol), PdCl₂(PPh₃)₂ (18 mg, 0.025 mmol) and copper iodide (10 mg, 0.051 mmol) were added to degassed THF (15 mL) and stirred at room temperature for two minutes. 4-ethynylaniline (294 mg, 2.51 mmol) was added to the solution followed by a dropwise addition of 0.5 M aqueous ammonia (10 mL), leaving to stir overnight at room temperature under nitrogen. Upon completion, the mixture was extracted with diethyl ether (3 x 30 mL), dried over MgSO₄, filtered and the solvents were evaporated. The product was purified via column chromatography (10% EtOAc in hexane), to afford a brown powder (210 mg, 40%). The data matched that reported in literature.³⁵⁶

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.14 – 7.06 (m, 4H, H₁₊₅), 6.56 – 6.49 (m, 4H, H₂₊₄), 5.40 (s, 4H, (NH₂)₂). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ 148.67 (2C, NH₂-C), 132.02 (4C, C₁₊₅), 113.67 (4C, C₂₊₄), 113.59 (2C, ArC-C_{alkyne}), 109.52 (C alkyne), 87.75 (C alkyne). **HRMS** (ES⁺) calculated for C₁₄H₁₂N₂ [M+H]⁺ 209.1079 found 209.1079.

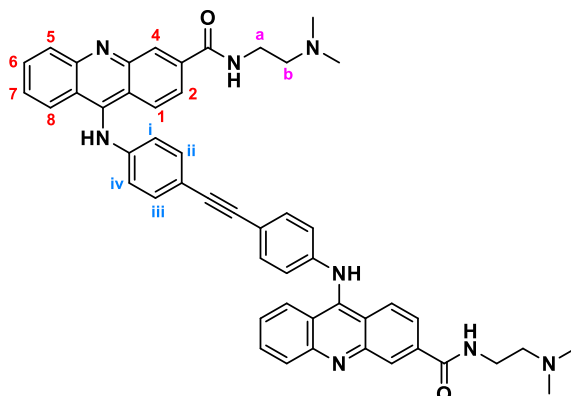
6.4.1.24 9,9'-(ethyne-1,2-diylbis(4,1-phenylene))bis(azanediyil))bis(N-(2-(dimethylamino) ethyl)acridine-4-carboxamide (**2.105**)



9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (100 mg, 0.30 mmol) and excess dry phenol (1.506 g) were heated to 110 °C and stirred for fifteen minutes. The solution was cooled to 55 °C and **2.104** (31 mg, 0.15 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (4 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (94 mg, 67%).

IR ν_{max} (neat) / cm⁻¹ 3334 (NH), 2720 (CH₂/CH₃), 1620 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.71 (d, *J* = 7.1 Hz, 2H), 8.48 (d, *J* = 8.7 Hz, 2H), 8.25 (d, *J* = 8.7 Hz, 2H), 8.11 – 8.01 (m, 4H), 7.67 (d, *J* = 8.2 Hz, 4H), 7.62 – 7.49 (m, 4H), 7.45 (d, *J* = 8.2 Hz, 4H), 3.96 (t, *J* = 5.4 Hz, 4H, H_a), 3.54 (t, *J* = 5.4 Hz, 4H, H_b), 3.05 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₅₀H₄₆N₈O₂ [M+H]⁺ 791.3744 found 791.3822. **UV** λ_{max} = 392 nm. **HPLC**: *t*_R = 19.7 min.

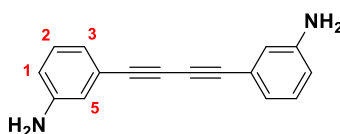
6.4.1.25 9,9'-(ethyne-1,2-diylbis(4,1-phenylene))bis(azanediyil))bis(N-(2-(dimethylamino ethyl)acridine-3-carboxamide (**2.106**)



9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (60 mg, 0.18 mmol) and excess dry phenol (1.804 g) were stirred at 110 °C for fifteen minutes. The solution was cooled to 55 °C and **2.104** (18 mg, 0.09) mmol was added, the reaction stirred overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (43 mg, 51%).

IR ν_{max} (neat) / cm⁻¹ 3395 (NH), 3250 (CH), 2945 (CH₂/CH₃), 1624 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.66 (dd, *J* = 1.7, 0.7 Hz, 2H), 8.25 (dt, *J* = 8.9, 1.0 Hz, 2H), 8.19 – 8.10 (m, 4H), 7.95 – 7.86 (m, 4H), 7.59 (ddd, *J* = 8.7, 6.6, 1.0 Hz, 2H), 7.36 – 7.28 (m, 4H), 7.09 (m, 2H), 6.92 – 6.84 (m, 4H), 3.63 (t, *J* = 6.8 Hz, 4H, H_a), 2.66 (t, *J* = 6.8 Hz, 4H, H_b), 2.36 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₅₀H₄₆N₈O₂ [M+H]⁺ 791.3822 found 791.3821. **UV** λ_{max} = 417 nm. **HPLC**: *t*_R = 16.8 min.

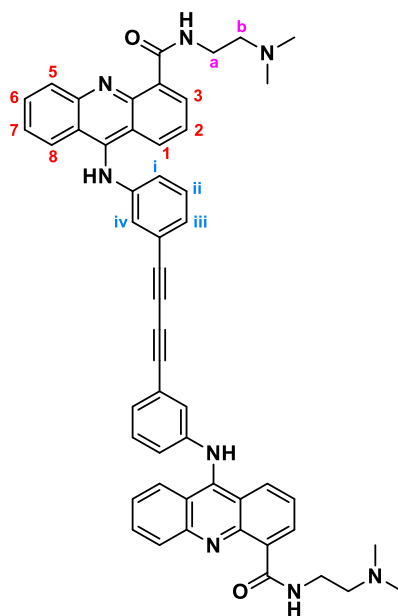
6.4.1.26 3,3'-(Buta-1,3-diyne-1,4-diyl)dianiline (**2.108**)



3-ethynylaniline (230 mg, 1.96 mmol) and $\text{Cu}(\text{OAc})_2$ (1.013 g, 5.5 mmol) were dissolved in a 1:1 mixture of MeOH and pyridine (v/v, 20 mL), was heated under reflux for 2 hours. Volatile solvents were evaporated off yielding a semi-solid, which was dissolved in water and extracted with EtOAc (3 x 10 mL), dried over MgSO_4 and filtered. Column chromatography (0 – 5% MeOH in CH_2Cl_2) afforded pure product as a brown solid (193 mg, 85%). The data matched that reported in literature.³⁵⁸

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 7.05 (t, J = 7.9 Hz, 2H, H_5), 6.74 – 6.61 (m, 6H, H_{1+2+3}), 5.31 (s, 4H, $(\text{NH}_2)_2$). **^{13}C NMR** (100 MHz, DMSO) δ 148.93 (2C, $\text{NH}_2\text{-C}$), 129.40, 120.68, 119.75, 116.62, 115.69, 82.31 (2C, C alkyne), 72.36 (2C, C alkyne). **HRMS** (ES+) calculated for $\text{C}_{16}\text{H}_{12}\text{N}_2$ $[\text{M}+\text{H}]^+$ 233.1079 found 233.1085.

6.4.1.27 9,9'-(buta-1,3-diyne-1,4-diylbis(3,1-phenylene))bis(azanediyl))bis(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.109**)

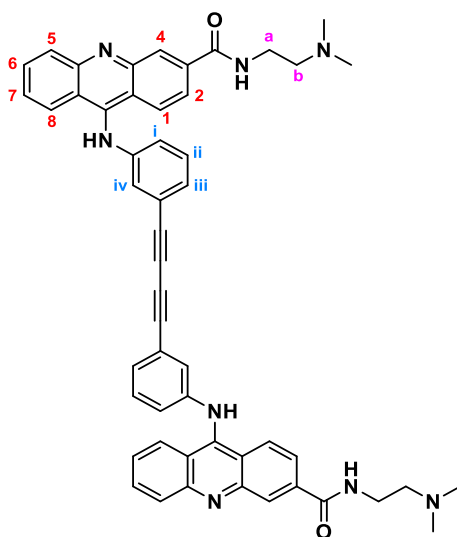


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (100 mg, 0.30 mmol) and excess dry phenol (1.508 g) were stirred at 110 °C for fifteen minutes. The solution was cooled to 55 °C and **2.108** (35 mg, 0.15 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in

EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (109 mg, 76%).

IR v_{max} (neat) / cm⁻¹ 3295 (NH), 3045 (CH), 2627 (CH₂/CH₃), 2765 (CH₂/CH₃), 1618 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.65 (d, *J* = 7.3 Hz, 2H), 8.52 (d, *J* = 8.6 Hz, 2H), 8.25 (d, *J* = 8.8 Hz, 2H), 8.17 – 8.04 (m, 4H), 7.69 (d, *J* = 8.6 Hz, 4H), 7.70 – 7.52 (m, 4H), 7.46 (d, *J* = 8.8 Hz, 4H), 3.95 (t, *J* = 5.7 Hz, 4H, H_a), 3.54 (t, *J* = 5.7 Hz, 4H, H_b), 3.06 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₅₂H₄₆N₈O₂ [M+H]⁺ 815.3822 found 815.3833. **UV** λ_{max} = 393 nm. **HPLC**: *t*_R = 20.0 min.

6.4.1.28 9,9'-(buta-1,3-diyne-1,4-diylbis(3,1-phenylene))bis(azanediyl))bis(*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.110**)



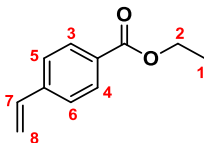
9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-3-carboxamide (**2.68**) (60 mg, 0.18 mmol) and excess dry phenol (1.506 g) were stirred at 110 °C for fifteen minutes. The

solution was cooled to 55 °C and **2.108** (21 mg, 0.09 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 30 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (109 mg, 76%).

IR ν_{max} (neat) / cm⁻¹ 3260 (NH), 2944 (CH), 2729 (CH₂/CH₃), 1623 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.64 (s, 2H), 8.22 (d, *J* = 8.9 Hz, 2H), 8.10 (dd, *J* = 15.8, 8.9 Hz, 4H), 7.88 (m, 4H), 7.64 – 7.52 (m, 2H), 7.34 – 7.25 (m, 4H), 7.12 – 7.03 (m, 2H), 6.85 (d, *J* = 8.4 Hz, 4H), 3.64 (t, *J* = 6.7 Hz, 4H, H_a), 2.72 (t, *J* = 6.7 Hz, 4H, H_b), 2.41 (s, 12H, (CH₃)₄). **HRMS** (ES⁺) calculated for C₅₂H₄₆N₈O₂ [M+H]⁺ 815.3822 found 815.3851. **UV** λ_{max} = 414 nm. **HPLC**: *t*_R = 17.2 min.

6.4.2 Chapter 3

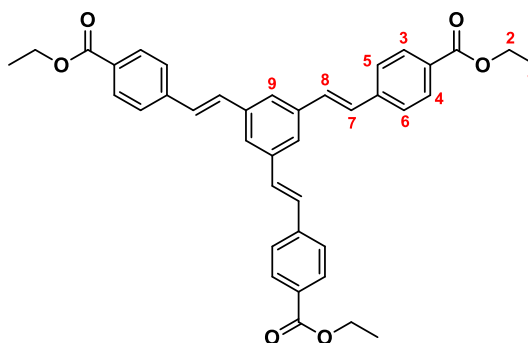
6.4.2.1 Ethyl 4-ethenylbenzoate (3.39)



4-vinylbenzoic acid (785 mg, 5.29 mmol), *N,N*-dicyclohexylcarbodiimide (DCC) (1.310 g, 6.34 mmol) and 4-dimethylaminopyridine (DMAP) (64 mg, 0.52 mmol) were dissolved in anhydrous CH_2Cl_2 . EtOH was added (3.08 mL, 52.90 mmol) and the reaction was stirred at room temperature overnight under nitrogen. The solution was filtered through a celite plug and further purified by column chromatography (0 – 30% EtOAc in Hexane), yielding a clear oil (839 mg, 90%). The data matched that reported in literature.⁴⁵²

IR ν_{max} (neat) / cm^{-1} 2981 (CH), 2854 (CH_2/CH_3), 1711 ($\text{C}=\text{O}$). **^1H NMR** (400 MHz, CDCl_3) δ 8.04 – 7.96 (m, 2H, H_{3+4}), 7.49 – 7.42 (m, 2H, H_{5+6}), 6.75 (dd, $J = 17.6, 10.9$ Hz, 1H, H_7), 5.86 (dd, $J = 17.6, 0.8$ Hz, 1H, $\text{H}_{8,1}$), 5.38 (dd, $J = 10.9, 0.8$ Hz, 1H, $\text{H}_{8,2}$), 4.37 (q, $J = 7.1$ Hz, 2H, H_2), 1.39 (t, $J = 7.1$ Hz, 3H, H_1).

6.4.2.2 4,4',4''-[1,3,5-benzenetriyltri-(1*E*)-2,1-ethenediyl]tris-, 1,1',1''-triethyl ester (3.36)

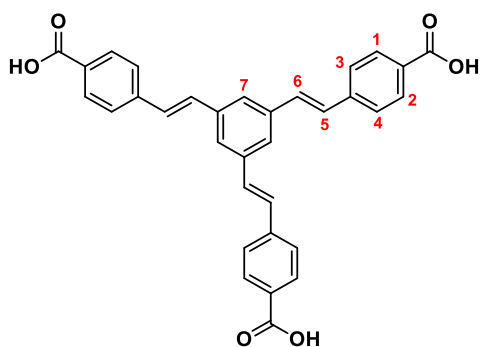


Tetrahydrofuran was degassed using a freeze-pump-thaw cycle (x3), to which 1,3,5-tribromobenzene (326 mg, 1.03 mmol), $\text{Pd}(\text{dba})_3\text{CHCl}_3$ (300 mg, 0.28 mmol), $[(t\text{-Bu})_3\text{P}]\text{BF}_4$ (181 mg, 0.62 mmol) and *N,N*-methyldicyclohexane (0.968 mL, 5.01 mmol) were added followed by **3.40** (603 mg, 3.42 mmol). The reaction was heated under reflux overnight. Upon completion, indicated by TLC (5% MeOH in CH_2Cl_2), the solvents were removed under vacuum and the crude material was redissolved in CH_2Cl_2 and filtered, removing any insoluble materials. Column chromatography (0 –

2% MeOH in CH₂Cl₂) was used for purification to afford pure product as a white powder (464 mg, 75%). The data matched that reported in literature.⁴⁵¹

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.03 – 7.96 (m, 6H, H₃₊₄), 7.89 (s, 3H, H₉), 7.85 – 7.74 (m, 6H, H₅₊₆), 7.56 – 7.42 (m, 6H, H₇₊₈), 4.33 (q, *J* = 7.1 Hz, 6H, H₂), 1.34 (t, *J* = 7.1 Hz, 9H, H₁). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ 165.48 (C=O), 141.65, 137.54, 131.30 (C alkene), 129.70 (C alkene), 128.73, 128.27, 126.67, 124.95, 60.70 (CH₂), 14.20 (CH₃). **HRMS** (ES+) calculated for C₃₉H₃₆O₆ [M+H]⁺ 601.2590 found 601.2585.

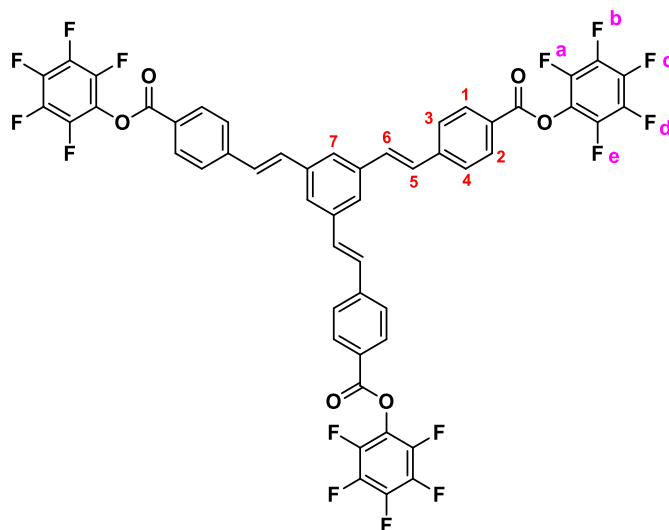
6.4.2.3 4,4',4''-(1,3,5-Benzenetriyltri-(1E)-2,1-ethenediyl)tris(benzoic acid) (**3.42**)



Compound **2.36** (333 mg, 0.55 mmol) and LiOH monohydrate (148 mg, 3.52 mmol) were dissolved in a 2 to 1 solution of water to THF (20 mL total) and the reaction was heated under reflux for 4 hours. On cooling, volatile solvents were removed under vacuum, the remaining solution was further diluted in water and acidified with 1 M HCl. The resulting precipitate was collected via filtration, yielding the product as a beige powder (256 mg, 90%). The data matched that reported in literature.⁴⁵¹

IR *v*_{max} (neat) / cm⁻¹ 3386 (CH), 2854 (CH₂/CH₃), 1685 (C=O). **¹H NMR** (400 MHz, DMSO-*d*₆) δ 8.01 – 7.95 (m, 6H, H₁₊₂), 7.88 (s, 3H, H₇), 7.79 – 7.73 (m, 6H, H₃₊₄), 7.56 – 7.42 (m, 6H, H₅₊₆). **¹³C NMR** (100 MHz, DMSO-*d*₆) δ 167.10 (C=O), 141.35, 137.64, 130.50 (C alkene), 129.95 (C alkene), 129.76, 128.47, 126.53, 125.03.

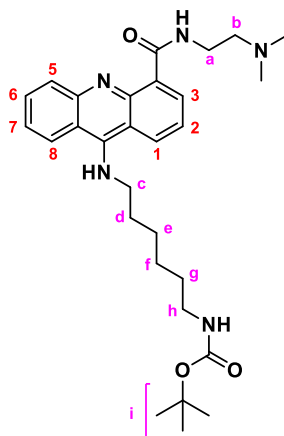
6.4.2.4 Tris(perfluorophenyl)-4,4',4''-((1E,1'E,1''E)-benzene-1,3,5-triyltris(ethene-2,1-diyl))tribenzoate (**3.43**)



Compound **3.42** (256 mg, 0.49 mmol) was dissolved in THF, the solution was cooled to 0 °C and EDC (1.133 g, 5.89 mmol) and DIPEA (2.05 mL, 11.78 mmol) was added. The reaction was allowed to reach room temperature, followed by addition of pentafluorophenol (1.08, 5.89 mmol), stirring at room temperature overnight. Column chromatography (CH₂Cl₂) afforded pure product as a white solid (323 mg, 65%). The data matched that reported in literature.⁴⁴⁸

IR ν_{max} (neat) / cm⁻¹ 3034 (CH), 2804 (CH₂/CH₃), 1759 (C=O). **¹H NMR** (400 MHz, CDCl₃) δ 8.27 – 8.20 (m, 6H, H₁₊₂), 7.76 – 7.69 (m, 9H, H₃₊₄₊₇), 7.41 – 7.26 (m, 6H, H₅₊₆). **¹⁹F NMR** (376 MHz, CDCl₃) -152.36 – -152.51 (m), -157.86 (t, J = 21.6 Hz), -162.09 – -162.44 (m).

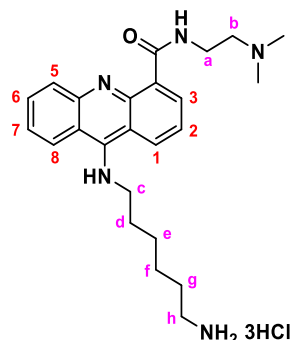
6.4.2.5 *Tert*-butyl 6-(4-(2-(dimethylamino)ethylcarbamoyl)acridin-9-ylamino)hexyl carbamate (**3.53**)



9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (200 mg, 0.60 mmol) and excess dry phenol (1.506 g) were stirred at 110 °C for fifteen minutes. The solution was cooled to 55 °C and *N*-Boc-,5-hexanediamine (197 mg, 0.91 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 5 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (252 mg, 83%). The data matched that reported in literature.³⁶⁷

¹H NMR (400 MHz, MeOD-*d*₄) δ 8.62 (dd, *J* = 7.2, 1.5 Hz, 1H, H₁), 8.41 (dd, *J* = 8.7, 1.5 Hz, 1H, H₃), 8.26 (dd, *J* = 8.9, 1.3 Hz, 1H, H₈), 7.94 (d, *J* = 8.7 Hz, 1H, H₅), 7.71 – 7.65 (m, 1H, H₆), 7.41 – 7.32 (m, 2H, H₂₊₇), 3.81 (t, *J* = 7.2 Hz, 2H, H_c), 3.72 (t, *J* = 6.5 Hz, 2H, H_a), 2.96 (t, *J* = 6.9 Hz, 2H, H_h), 2.72 (t, *J* = 6.5 Hz, 2H, H_b), 2.39 (s, 6H, (CH₃)₂), 1.77 (p, *J* = 7.2 Hz, 2H, H_d), 1.35 – 1.22 (m, 15H, H_{3+f+g+i}).

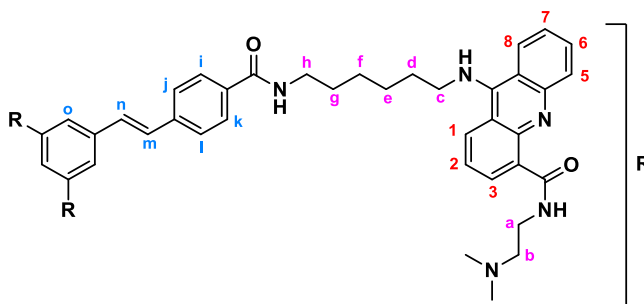
6.4.2.6 9-(6-Aminohexylamino)-N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (3.54)



Compound **3.53** (250 mg, 0.49 mmol) was stirred in 1.25 M HCl in MeOH (4 mL) at room temperature overnight. Solvents were evaporated under vacuum yielding the product as a yellow oil (247 mg, 98%).

IR ν_{max} (neat) / cm^{-1} 3371 (NH), 3226 (CH), 2935 (CH_2/CH_3), 1620 (C=O). **^1H NMR** (400 MHz, $\text{MeOD}-d_4$) δ 8.74 (d, $J = 8.7$ Hz, 1H, H_1), 8.61 – 8.52 (m, 2H, H_{3+8}), 8.01 (ddd, $J = 8.5, 6.8, 1.3$ Hz, 1H, H_6), 7.93 (dd, $J = 8.5, 1.3$ Hz, 1H, H_5), 7.70 – 7.58 (m, 2H, H_{2+7}), 4.18 (t, $J = 7.5$ Hz, 2H, H_h), 3.91 (t, $J = 5.8$ Hz, 1H, H_a), 3.52 (t, $J = 5.8$ Hz, 1H, H_b), 3.05 (s, 6H, $(\text{CH}_3)_2$), 2.93 (t, $J = 7.7$ Hz, 2H, H_c), 1.70 (p, $J = 7.7$ Hz, 2H, H_d), 1.56 – 1.48 (m, 6H, H_{e+f}). **HRMS** (ES+) calculated for $\text{C}_{24}\text{H}_{33}\text{N}_5\text{O}$ $[\text{M}+\text{H}]^+$ 408.2763 found 408.2764. The data matched that reported in literature.³⁶⁷

6.4.2.7 9,9',9''-(((4,4',4''-((1E,1'E,1''E)-benzene-1,3,5-triyltris(ethene-2,1-diyl))tris(benzoyl))tris(azanediyl))tris(hexane-6,1-diyl))tris(azanediyl))tris(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (3.38)

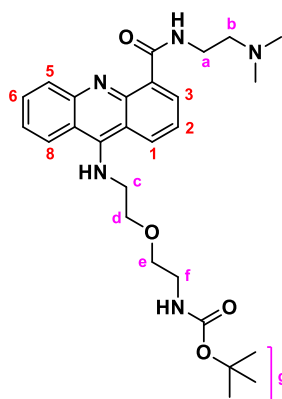


Compound **3.54** (150 mg, 0.37 mmol) and compound **3.43** (100 mg, 0.1 mmol) were dissolved in DMF (10 mL), DIPEA was added (0.203 mL, 1.2 mmol) and the reaction was stirred at room temperature overnight. Upon completion, water was added to the reaction, the resulting precipitate was collected and purified with column

chromatography (0 – 80% MeOH in EtOAc + 1% Et₃N). The final product was converted into the hexahydrochloride salt by stirring in 1.25 M HCl in MeOH (5 mL) for ten minutes, followed by precipitation with EtOAc. The precipitate was collected via filtration, yielding the final product as bright yellow solid (100 mg, 60%).

IR ν_{max} (neat) / cm⁻¹ 3236 (NH), 2954 (CH), 2629 (CH₂/CH₃), 1620 (C=O). **¹H NMR** (400 MHz, DMSO-*d*₆) δ 8.70 (s, 1H), 8.63 (d, *J* = 7.2 Hz, 1H), 8.53 (t, *J* = 5.6 Hz, 2H), 8.06 (d, *J* = 8.6 Hz, 1H), 7.92 – 7.80 (m, 4H), 7.70 (d, *J* = 8.1 Hz, 2H), 7.54 – 7.37 (m, 4H), 4.05 – 3.92 (m, 2H), 3.83 (q, *J* = 6.0 Hz, 2H), 3.34 (t, *J* = 6.0 Hz, 2H), 3.24 (d, *J* = 6.6 Hz, 2H), 2.82 (s, 6H), 1.87 (dd, *J* = 17.6, 10.5 Hz, 2H), 1.51 (q, *J* = 7.2 Hz, 2H), 1.38 (d, *J* = 12.6 Hz, 4H). **HRMS** (ES⁺) calculated for C₁₀₅H₁₁₇N₁₅O₆ [M+H]⁺ 1685.9420 found 1685.7037. **UV** λ_{max} = 417 nm. **HPLC**: *t*_R = 14.0 min.

6.4.2.8 *Tert*-butyl (2-(2-((4-((2-(dimethylamino)ethyl)carbamoyl)acridin-9-yl)amino)ethoxy)ethyl)carbamate (**3.56**)

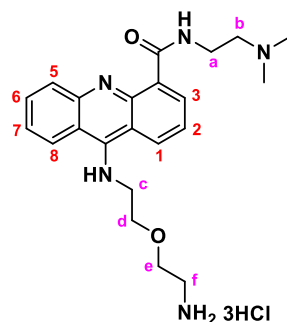


9-Chloro-*N*-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**2.63**) (190 mg, 0.58 mmol) and excess dry phenol (1.012 g) were stirred at 110 °C for fifteen minutes. The solution was cooled to 55 °C and *N*-Boc-2-(2-amino-ethoxy)-ethylamine (177 mg, 0.86 mmol) was added, the reaction was left to stir overnight. TLC confirmed the formation of the product (50:50 MeOH in EtOAc + 1% Et₃N). On cooling to room temperature 2 M NaOH (20 mL) was added to the reaction and poured into a separating funnel, extracted with CH₂Cl₂. The organic layer was separated, and the aqueous layer was extracted successively with CH₂Cl₂ (3 x 10 mL). The organic layers were combined, dried over MgSO₄ and filtered into a round-bottom flask. The solvent was removed under vacuum to yield an orange oil. Column chromatography (0 - 5 % MeOH in EtOAc + 1% Et₃N) was carried out, yielding the desired product as a bright red oil. The free base was converted into the tetrahydrochloride salt through the

addition of 1.25 M HCl in MeOH (3 mL) and the solution was stirred for ten minutes. The product was precipitated with EtOAc and collected through filtration and the bright yellow compound was finally freeze dried overnight, removing any traces of water (224 mg, 78%).

¹H NMR (400 MHz, MeOD-*d*₄) δ 8.61 (dd, *J* = 7.2, 1.5 Hz, 1H, H₁), 8.50 – 8.41 (m, 1H, H₃), 8.33 – 8.25 (m, 1H, H₈), 7.99 – 7.91 (m, 1H, H₅), 7.76 – 7.68 (m, 1H, H₆), 7.46 – 7.36 (m, 2H, H₂₊₇), 3.99 (t, *J* = 5.6 Hz, 2H, H_c), 3.77 – 3.65 (m, 4H, H_{d+e}), 3.48 (t, *J* = 5.2 Hz, 2H, H_a), 3.28 – 3.13 (m, 2H, H_f), 2.76 (t, *J* = 5.2 Hz, 2H, H_b), 1.39 (s, 9H, (CH₃)₃).

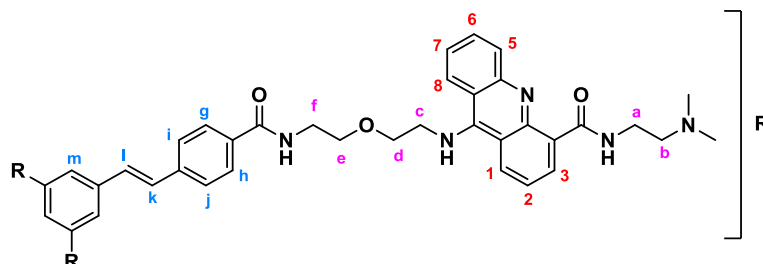
6.4.2.9 9-(2-(2-aminoethoxy)ethyl)amino)-N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**3.57**)



Compound **3.57** (220 mg, 0.44 mmol) was stirred in 1.25 M HCl in MeOH (4 mL) at room temperature overnight. Solvents were evaporated under vacuum yielding the product as a yellow oil (210 mg, 95%).

IR *v*_{max} (neat) / cm⁻¹ 3374 (NH), 3200 (CH), 2967 (CH₂/CH₃), 1619 (C=O). **¹H NMR** (400 MHz, MeOD-*d*₄) δ 8.84 (d, *J* = 8.6 Hz, 1H, H₁), 8.66 – 8.57 (m, 2H, H₃₊₈), 8.01 (ddd, *J* = 8.1, 6.8, 1.1 Hz, 1H, H₅), 7.92 (dd, *J* = 8.6, 1.2 Hz, 1H, H₆), 7.70 – 7.57 (m, 2H, H₂₊₇), 4.43 (t, *J* = 5.1 Hz, 2H, H_f), 4.10 (t, *J* = 6.9 Hz, 2H, H_c), 3.92 (dd, *J* = 6.9, 4.5 Hz, 2H, H_d), 3.82 (dd, *J* = 5.1, 4.5 Hz, 2H, H_e), 3.54 (t, *J* = 5.7 Hz, 2H, H_a), 3.19 – 3.11 (m, 2H, H_b), 3.06 (s, 6H, (CH₃)₂). **HRMS** (ES⁺) calculated for C₂₂H₂₉N₅O₂ [M+H]⁺ 396.2400 found 396.2407.

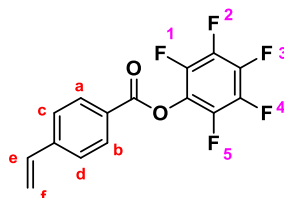
6.4.2.10 9,9',9''-((((4,4',4''-((1E,1'E,1''E)-benzene-1,3,5-triyltris(ethene-2,1-diyl))tris(benzoyl))tris(azanediy))tris(ethane-2,1-diyl))tris(oxy))tris(ethane-2,1-diyl))tris(azanediy))tris(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (**3.55**)



Compound **3.57** (100 mg, 0.25 mmol) and compound **3.43** (60 mg, 0.06 mmol) were dissolved in DMF (10 mL), DIPEA was added (0.123 mL, 0.72 mmol) and the reaction was stirred at room temperature overnight. Upon completion, water was added to the reaction, the resulting precipitate was collected and purified with column chromatography (0 – 80% MeOH in EtOAc + 1% Et₃N). The final product was converted into the hexahydrochloride salt by stirring in 1.25 M HCl in MeOH (5 mL) for ten minutes, followed by precipitation with EtOAc. The precipitate was collected via filtration, yielding the final product as bright yellow solid (61 mg, 62%).

IR ν_{max} (neat) / cm⁻¹ 3236 (NH), 3052 (CH), 2984 (CH₂/CH₃), 1622 (C=O). **¹H NMR** (400 MHz, DMSO-*d*₆) δ 8.62 (dd, *J* = 7.0, 1.4 Hz, 1H), 8.57 (d, *J* = 8.6 Hz, 1H), 8.51 (t, *J* = 5.7 Hz, 1H), 8.48 – 8.39 (m, 1H), 7.99 (d, *J* = 8.6 Hz, 1H), 7.88 – 7.81 (m, 3H), 7.81 – 7.73 (m, 1H), 7.68 (d, *J* = 8.1 Hz, 2H), 7.49 – 7.32 (m, 5H), 4.03 (d, *J* = 5.6 Hz, 2H), 3.80 (t, *J* = 5.4 Hz, 2H), 3.60 – 3.53 (m, 2H), 3.41 (d, *J* = 5.4 Hz, 2H), 2.30 (s, 6H). **HRMS** (ES⁺) calculated for C₁₀₀H₁₀₆N₁₄O₉ [M+H]⁺ 1649.8376 found 1649.8396. **UV** λ_{max} = 413 nm. **HPLC**: *t*_R = 14.0 min.

6.4.2.11 4-ethenyl-, 2,3,4,5,6-pentafluorophenyl ester (**3.59**)

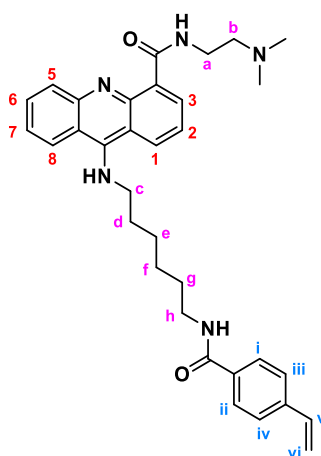


Compound **3.39** (256 mg, 0.49 mmol) was dissolved in THF, the solution was cooled to 0 °C and EDC (1.130 g, 5.89 mmol) and DIPEA (2.05 mL, 11.78 mmol) was added. The reaction was allowed to reach room temperature, followed by addition of

pentafluorophenol (1.08, 5.89 mmol), stirring at room temperature overnight. Column chromatography (CH₂Cl₂) afforded pure product as a white solid (323 mg, 65%).

¹H NMR (400 MHz, CDCl₃) δ 8.16 (d, *J* = 8.2 Hz, 2H), 7.56 (d, *J* = 8.2 Hz, 2H), 6.80 (dd, *J* = 17.6, 10.9 Hz, 1H), 5.94 (d, *J* = 17.6 Hz, 1H), 5.48 (d, *J* = 10.9 Hz, 1H). **¹⁹F NMR** (376 MHz, CDCl₃) -152.36 – -152.51 (m), -157.86 (t, *J* = 21.6 Hz), -162.09 – -162.44 (m).

6.4.2.12 9-ethene-2,1-diyl(benzoyl)(azanediyl)(hexane-6,1-diyl)(azanediyl)-(N-(2-(dimethylamino)ethyl)acridine-4-carboxamide (3.58)



Compound **3.54** (70 mg, 0.13 mmol) and compound **3.43** (12.5 mg, 0.04 mmol) were dissolved in DMF (10 mL), DIPEA was added (0.269 mL, 1.3 mmol) and the reaction was stirred at room temperature overnight. Upon completion, water was added to the reaction, the resulting precipitate was collected and purified with column chromatography (0 – 80% MeOH in EtOAc + 1% Et₃N). The final product was converted into the hexahydrochloride salt by stirring in 1.25 M HCl in MeOH (5 mL) for ten minutes, followed by precipitation with EtOAc. The precipitate was collected via filtration, yielding the final product as bright yellow solid (13 mg, 60%).

¹H NMR (400 MHz, MeOD-*d*₄) δ 8.69 (d, *J* = 8.1 Hz, 1H), 8.61 (s, 1H), 8.50 (d, *J* = 8.7 Hz, 1H), 7.99 (t, *J* = 7.8 Hz, 1H), 7.89 (d, *J* = 8.4 Hz, 1H), 7.74 (d, *J* = 8.2 Hz, 2H), 7.60 (dd, *J* = 17.8, 9.9 Hz, 2H), 7.48 (d, *J* = 8.2 Hz, 2H), 6.77 (dd, *J* = 17.7, 11.0 Hz, 1H), 5.88 (d, *J* = 17.7 Hz, 1H), 5.34 (d, *J* = 11.0 Hz, 1H), 4.15 (t, *J* = 7.2 Hz, 2H), 3.94 – 3.88 (m, 2H), 3.54 – 3.46 (m, 2H), 3.41 – 3.34 (m, 2H), 3.04 (s, 6H), 2.06 – 1.97 (m, 2H), 1.66 (m, *J* = 7.0 Hz, 2H), 1.58 – 1.46 (m, 4H). **LRMS** (ES⁺) calculated for C₃₃H₃₉N₅O₂ [M+H]⁺ 538.3 found 538.4.

Chapter 6

References

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